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The Program BATTLE seeks to establish individualized targeted therapy by prospectively examining patients' tumor biomarker profiles and assigning them to corresponding targeted therapies with the expectation to yield a better clinical outcome. Based on common altered signaling pathways in lung cancer, the BATTLE Program proposes to develop four phase II trials for chemorefractory, advanced NSCLC patients: erlotinib, ZD6474, bexarotene with erlotinib, and sorafenib which target, respectively, EGFR, VEGF / VEGFR, retinoid X receptor and cyclin D1, and Ras / Raf signaling pathways. A novel adaptive randomization statistical design will be applied to the clinical trials to accelerate the identification of best-fit treatment for patients. We propose also to study the molecular mechanisms of response or resistance to these targeted agents, identify novel molecular features in tumors and surrogate tissues to correlate with tumor response or resistance to the agents and, finally, explore other novel targeted agents (RAD001 and perifosine) in combination and their mechanisms of action by targeting mTOR and PI3K/Akt signaling, and develop phase I trials to test these combinations.

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Lung cancer, biomarker, targeted therapy, ZD6474, erlotinib, Sorefenib, bexatene

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INTRODUCTION

Lung cancer is the leading cause of cancer-related death in both men and women in the United States. Chemotherapy has reached its limit in improving the survival of lung cancer patients. Therefore, a different strategy must be waged in the battle against lung cancer. Targeted therapy, a newly emerged therapeutic approach in lung cancer, has succeeded in some cancer types and demonstrated its initial success in the treatment of lung cancer when a class of targeted agents termed epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors, such as gefitinib and erlotinib, improved tumor response rates in patients with advanced non-small cell lung cancer (NSCLC), which was strongly correlated to the presence of *EGFR* mutations in the tumors (Cappuzzo and Hirsch et al., 2004; Cappuzzo and Magrini et al., 2004; Gatzemeier et al., 2004; Herbst and Giaccone et al., 2004; Herbst and Prage et al., 2004; Herbst and Sandler et al., 2004; Lynch et al., 2004; Kobayashi et al., 2005; Miller et al., 2004; Pao et al., 2004; Paez et al., 2004; Shepherd et al., 2004; Shigematsu et al., 2005). This has for the first time demonstrated the importance of selecting patients for individualized targeted therapy in NSCLC.

The Program BATTLE (Biomarker-integrated Approaches of Targeted Therapy for Lung Cancer Elimination) seeks to establish individualized targeted therapy by prospectively examining patients' tumor biomarker profiles and assigning them to corresponding targeted therapies with the expectation to yield a better clinical outcome. This novel approach will be a proof-of-principle experiment to test the benefit of molecular-based individualized targeted therapy for lung cancer patients. Specifically, the objectives of the BATTLE program are:

1) To establish a clinical trial program using biomarkers to select individualized targeted therapy for patients with chemorefractory advanced NSCLC through the implementation of molecular classification based on the status of specific targeted biomarkers and adaptive randomization via hierarchical Bayes modeling.
2) To study the molecular mechanisms of response and resistance to targeted agents to discover new signaling pathways for test in future trials.
3) To identify molecular features in tumor tissues to correlate with tumor response or resistance, and identify serum biomarkers as surrogates.
4) To investigate other targeted agents in combination to overcome the resistance due to novel signaling pathways (e.g., mTOR and PI3K/Akt) and improve treatment efficacy.

BATTLE is composed of four Specific Aims with four phase II clinical trials and an umbrella protocol in Aim 1, six research projects in Aims 2 - 4, and two potential phase I trials in Aim 4. Here, we present our scientific progression the BATTLE program for the first grant year.

PROGRESS REPORT (Body)

Aim 1 To establish a clinical trial program using biomarker assessment to select individualized targeted therapy for previously treated chemorefractory advanced NSCLC patients.

(PI, Co-PIs, and Investigators: Drs. Waun Ki Hong, Roy Herbst, Edward S. Kim, George Blumenschien, Ann Tsao, Hai Tran, Marshall Hicks, Rodolfo Morice, Bruce Johnson)

Specific Aim 1 has five clinical trials: one umbrella trial and four Phase II open-label trials. After screening, eligible patients will be enrolled in the umbrella trial, and tumor biopsies will be taken for biomarker analysis conducted by the Biomarker Core. (For details, please see the Biomarker

Core section of this report.) Biomarker results will be analyzed by the Biostatistics and Data Management Core. (For details, please see the Biostatistics and Data Management Core section of this report.) There are two components of this study: 1) an equal randomization phase, where patients are randomized equally to the four trials after biomarker analysis; and 2) an adaptive randomization phase, where patients are to one of the four clinical trials based on their tumor biomarker characteristics. The four Phase II clinical trials are presented in the four sub-aims of Aim 1 described below.

Aim 1.1 To conduct a clinical trial with erlotinib in patients with previously treated advanced NSCLC whose tumors have EGFR mutations and / or overrepresentation.

Primary objective is to determine the 8-week progression-free survival (PFS) rate of patients with previously treated advanced NSCLC whose tumors have EGFR mutations and / or overrepresentation who are treated with erlotinib

Secondary objectives are to 1) determine the overall survival rate, response rate, and toxicity profiles of patients with advanced NSCLC whose tumors have EGFR mutations and / or overrepresentation and treated with erlotinib, 2) determine the plasma and (if available) tumor tissue concentrations of erlotinib and their correlation with response and toxicity by using pharmacokinetics and pharmacodynamic modeling.

Aim 1.2 To conduct a clinical trial with ZD6474 in patients with previously treated advanced NSCLC whose tumors have increased VEGF and / or VEGFR-2.

Primary objective is to determine the 8 - week PFS rate in patients with previously treated advanced NSCLC whose tumors have increased VEGF and / or VEGFR-2 who are treated with ZD6474.

Secondary objectives are to 1) determine the overall survival rate, response rates, and toxicity profiles of patients with advanced NSCLC whose tumors express increased VEGF and / or VEGFR-2 and treated with ZD6474, and 2) determine the plasma and (if available) tumor tissue levels of ZD6474 and their correlations with response and toxicity by using pharmacokinetics and pharmacodynamic modeling.

Aim 1.3 To conduct a clinical trial with the combination of bexarotene and erlotinib trial in patients with previously treated advanced NSCLC whose tumors have expressed RXRs and / or increased cyclin D1.

Primary objective is to determine the 8 - week PFS rate in patients with previously treated advanced NSCLC whose tumors have expressed RXRs and / or increased cyclin D1 who are treated with the combination of Bexarotene and Erlotinib.

Secondary objectives are to 1) determine the overall survival rate, response rate, and toxicity profiles of patients with advanced NSCLC whose tumors have expressed RXRs and / or increased cyclin D1 and treated with the combination of bexarotene and erlotinib, 2) determine the plasma and (if available) tumor tissue concentrations of bexarotene and erlotinib and their correlation with response and toxicity by using pharmacokinetics and pharmacodynamic modeling.

Aim 1.4 To conduct a clinical trial with sorafenib trial in patients with previously treated advanced NSCLC whose tumors have mutated *K-ras* and / or *B-raf.*

Primary objective is to determine the 8-week PFS rate in patients with previously treated advanced NSCLC whose tumors have mutant *K-ras* and / or *B-raf* who are treated with sorafenib.

Secondary objectives are to 1) determine the overall survival rate, response rate, and toxicity profiles of patients with advanced NSCLC whose tumors have mutated K-ras and / or B-raf and treated with sorafenib, 2) determine the plasma and (if available) tumor tissue concentrations of sorafenib and their correlation with response and toxicity by using pharmacokinetics and pharmacodynamic modeling.

Update: Summary of Specific Aim 1

Considering the highly interactive nature of the clinical trials in the BATTLE program, we will report the progress of the all the clinical trials in an integrated way.

The five BATTLE clinical protocols went through an extensive and comprehensive process of the protocol review, revision and approval by M. D. Anderson Cancer Center (MDACC) Clinical Research Committee (CRC), MDACC Institutional Research Board (IRB), the Department of Defense (DoD), Food and Drug Administration (FDA), and four pharmaceutical companies. On November 27, 2007, the protocols were activated for patient enrollment, less than eight months after the BATTLE grant was activated (April 1, 2006). This timeline is the shortest for protocol review, approval, and activation in our Department for a large multidisciplinary, integrated multi-trial program This rapid activation can be attributed to our departmental effort and, specifically, due to the dedication of our medical oncologists, protocol coordinator, research nurses, Biostatistician Core, Biomarker Core, and our institutional regulatory personnel.

Since activation, patient accrual has been excellent (Table 1). Response to our medical oncologists, interventional radiologists, and research nurses introduction of the BATTLE trials has been overwhelmingly. In addition, patients are highly interested and inquiring independently regarding the program. The coordination between the surgeons, medical oncologists, Biomarker Core and Biostatistics Core, has been well-planned and the clinical trials have been running very smoothly.

Biopsies for biomarkers have been obtained in over 85% of patients. Clinical data and tissues from the patients have been and continue to be collected for patient stratification into specific trials. These tissues will be also distributed to support research projects in the BATTLE grant.

By April 21, 2007, 63 patients were registered, with a total of 38 patients randomized, 34 with and 4 without complete biomarker information (Table 1 and Figure 1). The four patients without complete biomarker information were due to fibrosis and necrotic tumor tissue. Patients are approximately equally distributed between male and female (Figure 1). The clinical trials, erlotinib, ZD6474, erlotinib + bexarotene, and sorafenib, have enrolled 9, 9, 10, and 10 patients, respectively (Table 2). Of the 38 patients enrolled, 9 are in their first cycle of treatment, 9 have entered their second cycle, 3 are in cycle 3, 2 are in cycle 4, and 15 have completed the study.

Enrollment is ongoing. No significant adverse events have been reported for these treatment studies at this time.

For our collaboration with Dana Farber Cancer Institute (DFCI), we have sent the final approved protocols to Dr. Bruce Johnson. Currently, these protocols are under review by their IRB, and we hope to open the trials at DFCI later this year.

Table 1. Patient Accrual to BATTLE Protocols (April 21, 2007).

Year	Month	Accrual	Randomized W/ BMG	Randomized w/o BMG
2006	November	1	0	0
	December	11	5	0
2007	January	12	4	2
	February	13	8	0
	March	13	11	1
	April	13	6	1
	Total	63	34	4

Figure 1. Patient Demographics and Progress in BATTLE Protocols (April 21, 2007).

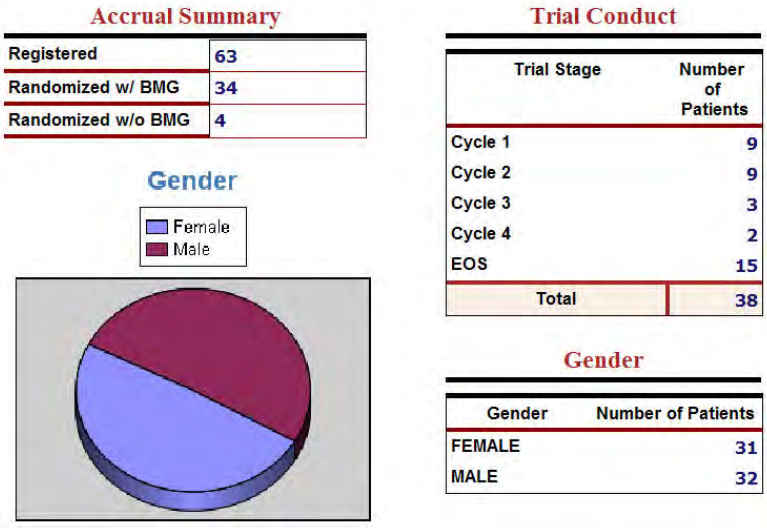


Table 2. Assignment of Patients to Biomarker Groups in BATTLE Protocols (April 21, 2007).

	Marker Group							
	1	2	3	4	5	Total	w/o BMG	Total
Tarceva	3	0	5	0	0	8	1	9
ZD6474	7	1	1	0	0	9	0	9
Tarceva and Targretin	6	2	1	0	0	9	1	10
Sorafenib	2	1	3	2	0	8	2	10
Total	18	4	10	2	0	34	4	38

Key Research Accomplishments:

- Activated all five BATTLE clinical protocols at M. D. Anderson Cancer Center within the first grant year.
- 63 patients registered and 38 randomized into one of the four treatment arms.
- Patient accrual and interest continue at a healthy pace.

- The success rate of the tissue acquisition and biomarker evaluation is over ~85%.
- Demonstrated highly efficient collaboration of Clinical teams, Biostatistics Core, and Biomarker Core.
- Developed the largest translational research program requiring core biopsy samples ever run in our department and, possibly, in the country.

Reportable Outcomes:

Presentations

- Herbst R, Lee JJ. The BATTLE Project. Presentation to the M. D. Anderson National Cancer Institute Cancer Center Support Grant External Advisory Board. Houston, Texas; January 2007.

Abstracts

- Zhou X, Kim ES, Herbst RS, Liu S, Wistuba II, Mao L, Lewis J, Lippman SM, Hong WK, Lee JJ. A clinical trial design applying Bayesian adaptive randomization for targeted therapy development in lung cancer - A step toward personalized medicine. Submitted to American Society of Clinical Oncology (ASCO) Annual Meeting, Atlanta, Georgia; June 2007.
- Liu S, Kim ES, Zhou X, Wistuba II, Herbst RS, Lewis J, Lee JJ. An Application of Adaptive Randomization Using Hierarchical Bayes Model in a Prospective Biomarker-Based Clinical Trial. Submitted to the Joint Statistical Meeting, Salt Lake City, Utah; August 2007.

Conclusions and Future Work:
The completion of the clinical trials is the key to this BATTLE research program. In the first grant year, the program has been significantly ahead of our proposed timeline. The trial accrual is reflective of the goals of the department in its completion. The design and innovative nature of the trials will keep interest high among patients who are treated at M. D. Anderson. Accrual is ongoing and will help support the other BATTLE specific aims with tumor response data, tissue specimens, and biomarker information.

Specific Aim 2: To investigate molecular mechanisms of response and resistance to the targeted agents used in the BATTLE program.

Specific Aim 2.1. To validate the molecular mechanisms of response and resistance to erlotinib for patients with chemorefractory NSCLC.

(PI and Co-PI: Bruce Johnson, M.D., and Pasi Jänne, M.D., Ph.D.)

The association between somatic *EGFR* mutations and clinical response to gefitinib in patients with (NSCLC was published in 2004). This proposal will further characterize *EGFR* mutations in subjects' tumors and tumor cell lines, and the relationship of these mutations, subject outcome, and *in vitro* behavior to different EGFR inhibitors. The data generated demonstrates that NSCLC patients with *EGFR* mutations identified in their tumors, typically respond to single-agent therapy with gefitinib treatment for a median of 1 year or longer, achieve a median overall survival of more than 2 years. This survival duration is 3-fold longer than that achieved with conventional chemotherapy in previously untreated subjects with NSCLC. Patients with increased copy number as determined by fluorescence *in situ* hybridization (FISH) who are

treated with gefitinib or erlotinib have a response rate of 20-30% and live a median of approximately 2 years. The goal of this research is to confirm these initial observations in prospective cohorts of subjects with NSCLC and somatic *EGFR* mutations or increased copy number with erlotinib as the initial therapy. This study will generate translational information on somatic mutations and copy number, prospective validation of the outcome of patients with NSCLC and *EGFR* mutations or increased copy number treated with erlotinib, information on the activation of the EGFR pathway in NSCLC and NSCLC cell lines, and information about mechanisms of resistance.

Objective 1: Establish estimates of the response and outcome of previously treated patients with prospectively identified somatic *EGFR* mutations treated with erlotinib.

Update

At this point, there have been nine patients accrued to the erlotinib arm of the BATTLE trials. The analyses for EGFR mutations, copy number, and immunohistochemistry (IHC) are being done by the BATTLE Biomarker Core to correlate the laboratory findings with the clinical outcome. For details, please refer to the Biomarker Core section of this report.

Objective 2: Determine effects of TGF-α, EGF, and Amphiregulin (AR) on the growth of *EGFR* mutant and wild type cell lines.

Update

Amphiregulin (AR) expression in medium

We analyzed the level of the ligands secreted (amphiregulin, EGF, TGF-α, and heregulin) in cell culture medium of 18 cancer cell lines using an ELISA assay (12 NSCLCs: PC-9, HCC827, HCC4006, H3255, Calu-3, H1648, H1437, HCC15, HCC193, HCC95, H661, and H2126; 2 bronchioloalveolar cell lines: H1666, H358; 4HNSCC: HN11, HN12, HN13 and HN28). Four NSCLC cell lines contain mutant *EGFR* (E746_A750del in PC-9 cells, E746_A750Adel in HCC827 cells, L747_E749del in HCC4006 cells, L858R in H3255 cells). The other cell lines studied all have wild-type *EGFR*. Seventeen of 18 tumor cell lines have wild-type *K-ras* gene; H358 has a *K-ras* mutation. Cell culture medium was harvested 48 hours after serum free medium was changed in confluent cells. Fourteen of the lung cancer and head and neck cancer cell lines tested with wild-type EGFR cell lines expressed various level of AR (from undetectable to 1600 pg pg/mL). The lung cancer cell lines with mutated EGFR produced less than 50 pg/mL of AR. Two NSCLC cell lines produced abundant TGF-α (H1666: >100 pg/mL and Calu-3: about 5 pg/mL). EGF is produced at detectable levels in some cell lines (HN11, H1666, HN12, H358, HN13 and H661). Previous reports have shown that concentrations of AR in excess of 250 pg/ml cause proliferation of murine kertinocytes. Therefore, we estimate the concentration of 250 pg/mL is likely to cause growth. We can thus divide wild-type *EGFR* lung cancer and head and neck cancer cell lines into two groups, AR high or low-expressing groups (Figure 2).

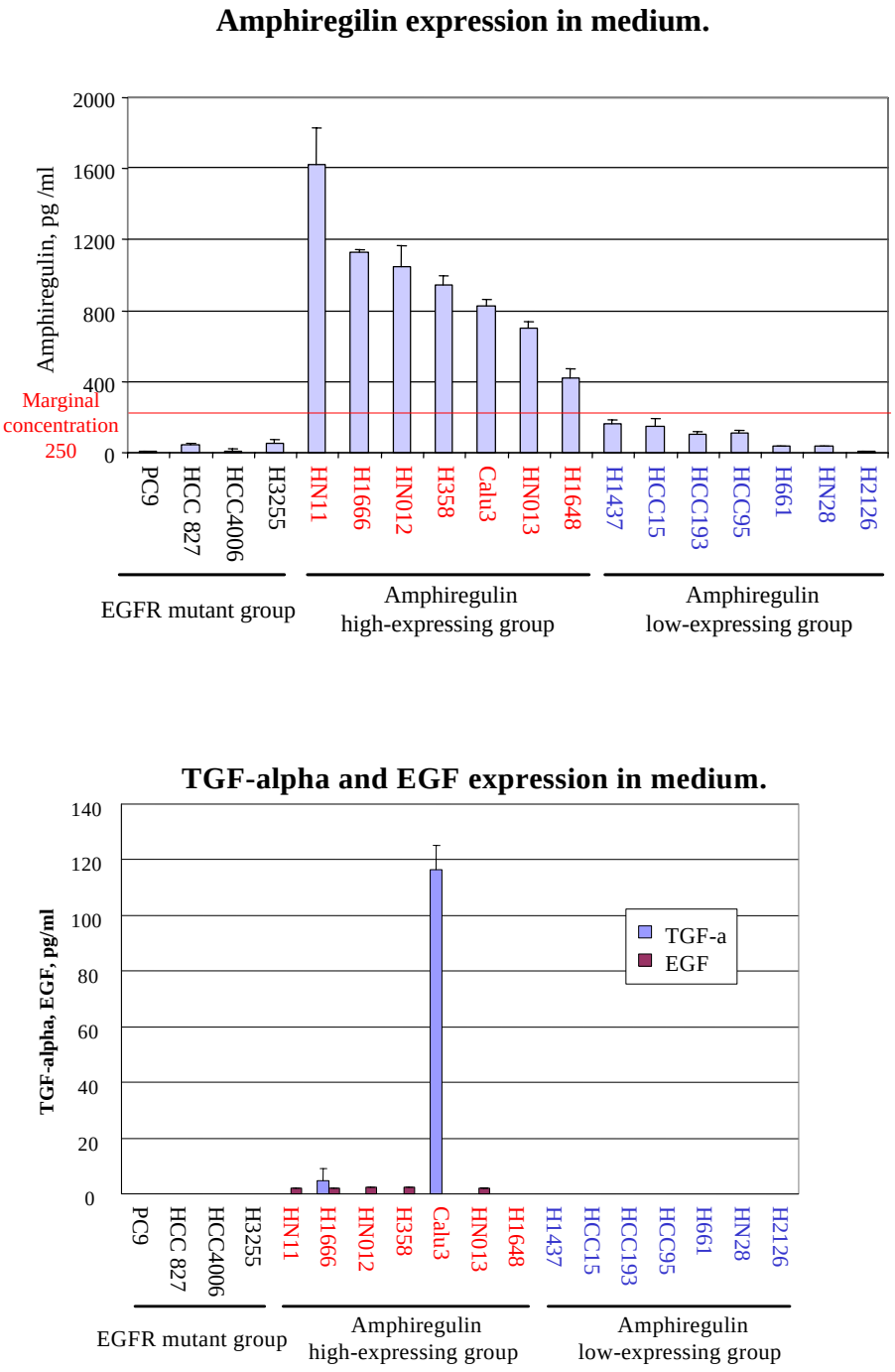
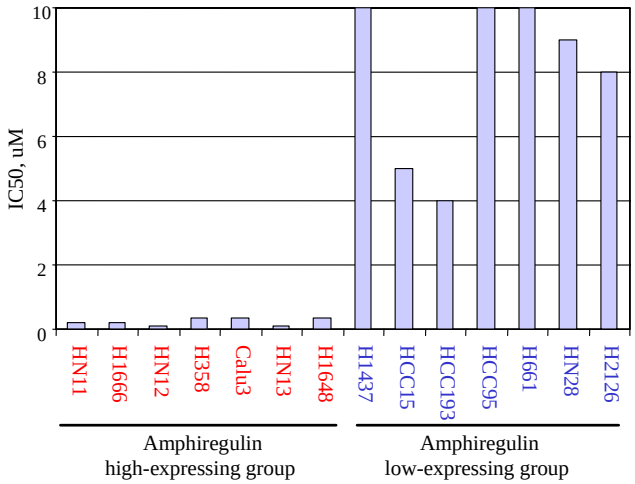


Figure 2. Expression of ligands (AR, EGF-alpha and EGF) in media of cancer cell lines.

The 14 cell lines with wild type EGFR underwent MTS assays (Promega, Madison, WI). Increasing concentrations of gefitinib were added and the IC_{50}s (i.e., the drug concentrations required for 50% inhibition of growth) were determined. The IC_{50}s of the 7 tumor cell lines (3 head and neck and 4 NSCLC) that produced amphiregulin in excess of 250 pg/ml were all less

than 1 μM. The gefitinib concentration of 1μM was selected because this is the steady state achievable plasma concentration for gefitinib. In contrast, the lung cancer and head and neck cell lines that made less than 250 pg/ml of amphiregulin were resistant to gefitinib with IC_{50}s in excess of 4 μM (Figure 3).

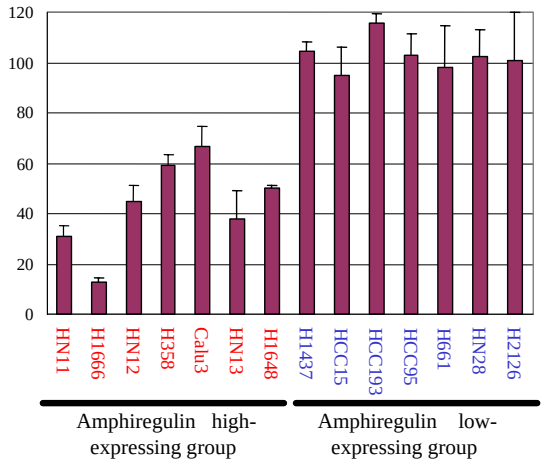
Figure 3.IC_{50} of cell lines treated with gefitinib (MTS assay).



Sensitivity to cetuximab

The dose-response curve of cetuximab displayed substantial growth inhibition at 0.033 mg/ml, and a plateau in the cell lines HN11, H1666 and HN13. The sensitivity to cetuximab up to 10 μg/ml in each cell line was determined. In the tumor cell lines producing AR in excess of 250 pg/ml, the viable cell count was less than 50% of the control at 10 μg/mL cetuximab, except Calu-3. In Calu-3, the growth inhibition by cetuximab was observed depending on the concentration, and viable cell count was approximately 40% of the control at 100 μg/mL. In contrast, the tumor cell lines producing less than 250 pg/ml of AR had no growth inhibition when up to 100 μg/mL cetuximab was added (Figure 4).

Figure 4. MTS assay of cell lines treated with 10 μg/ml cetuximab.

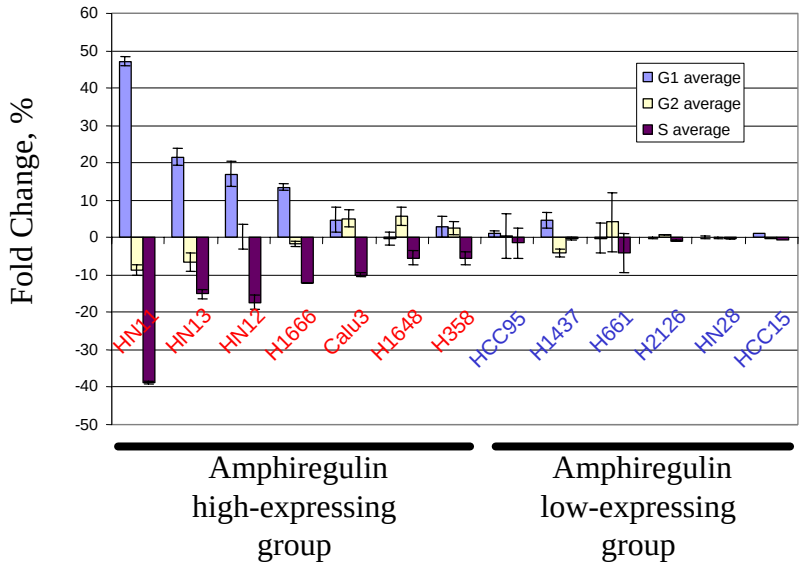


Objective 3: **Determine effects of TGF-α, EGF, and AR on the cell cycle and apoptosis of *EGFR* mutant and wild type cell lines.**

Update

The mechanism by which gefitinib and cetuximab inhibited growth were studied by analyzing the cell cycle distribution in cell lines following treatment with 1 μM gefitinib, 10 μg/mL cetuximab, and control cells (no drug) using flow cytometry. Both gefitinib and cetuximab induced cell-cycle arrest in G1 phase in all tumor cell lines producing an excess of 250 pg/ml of AR. Moreover, gefitinib induced a higher level of cell-cycle arrest compared to cetuximab in tumor cell lines producing 250 pg/ml of AR. in the tumor cell lines which produce less than 250 pg/ml of AR, neither gefitinib nor cetuximab had an effect on cell cycle except in HCC95. HCC95 was observed to significantly arrest in G1 phase by 1 μM of gefitinib; however, not by 10 μg/mL cetuximab (Figure 5).

Figure 5. Cell cycle analysis of cell lines treated with cetuximab.



Objective 4: **Determine effects of different *EGFR* mutations and EGFR inhibitors on phosphorylation of EGFR and downstream signaling intermediates.**

Update

The cellular response of tumor cell lines producing at least 250 pg/ml of AR and low AR producing cells (HN28 an (H661) were treated with gefitinib. As shown in Figure 6A-B, phosphor-EGFR was dramatically decreased when 0.1 μM gefitinib was added to HN11 and H1666 cells. There was also dramatic downregulation of phosphor-ERK1/2 and CyclinD1 when 0.1 or 1 μM gefitinib was added. Little change was observed in HN28 and H661 when gefitinib was added.

Figure 6A. **Western Blot of Cell lines expressing high-amphiregulin treated with gefitinib.**

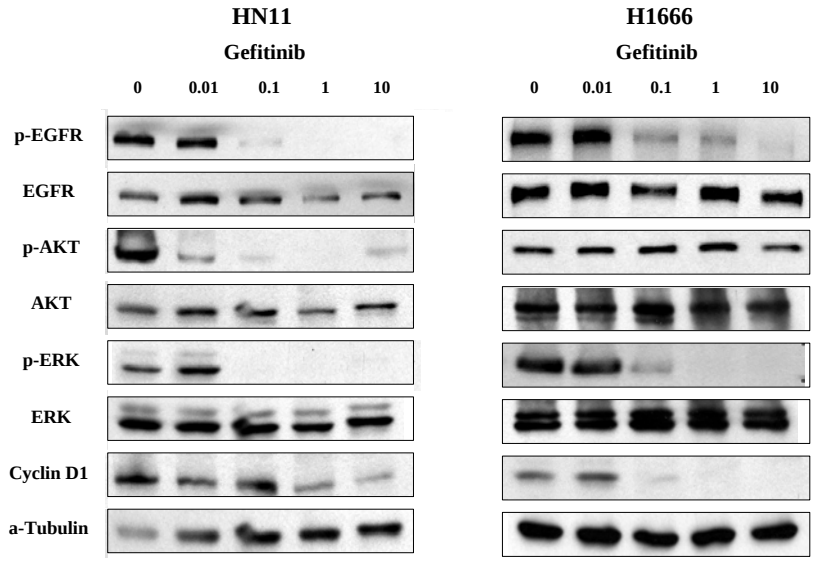
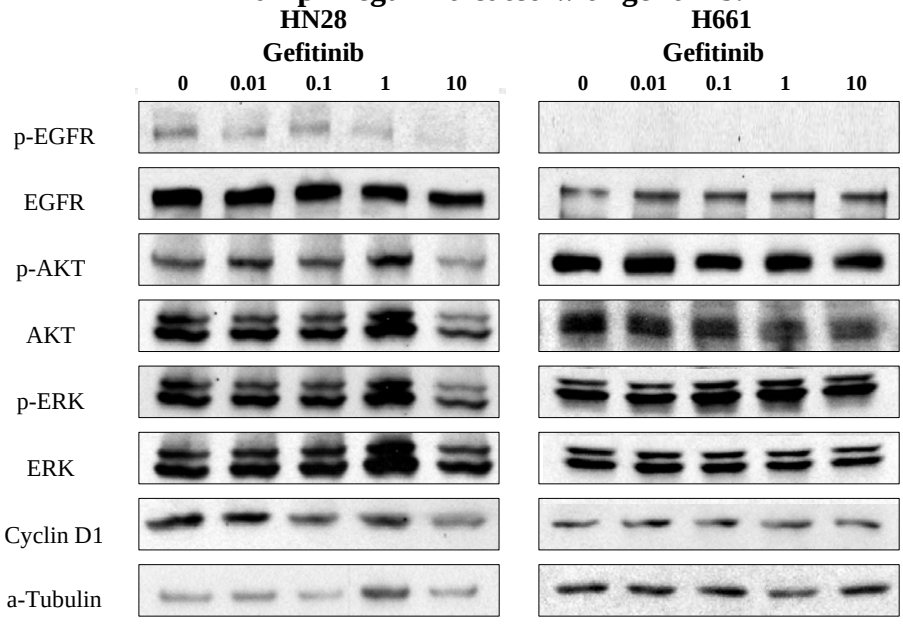


Figure 6B. **Western Blot of cell lines expressing low-amphiregulin treated with gefitinib.**



Key Research Accomplishments:

- Nine patients participating in the BATTLE trials have been assigned to the erlotinib trial.

- Amphiregulin (AR) is the agonist in tumor cell lines (both lung cancer and head and neck cancer cell lines) with wild type *EGFR* that is associated with response to gefitinib and cetuximab.

Reportable Outcomes:

Abstract

- Yonesaka K, Zejnullahu K, Homes AJ, Johnson, BE, Jänne PA. Presence of amphiregulin autocrine-loop predicts in vitro sensitivity of *EGFR* wild type NSCLC and HNSCC cell lines to gefitinib and cetuximab. Submitted to The World Conference of Lung Cancer, Seoul, Korea; September 2007.

Manuscript in submission

- Yonesaka K, Zejnullahu K, Homes AJ, Park JO, Koivunen J, Johnson BE, Jänne PA. Presence of amphiregulin autocrine-loop predicts in vitro sensitivity of *EGFR* wild type NSCLC and HNSCC cell lines to gefitinib and cetuximab. *Cancer Res*, 2007.

Conclusion:

We conclude that amphiregulin (AR) is the agonist in tumor cell lines (both lung cancer and head and neck cancer cell lines) with wild type EGFR that is associated with response to gefitinib and cetuximab. These agonists and the determination of whether EGFR is mutated will need to be studied in the tumor specimens from the patients participating in the BATTLE phase II trial of erlotinib to see if these *in vitro* findings translate into the clinical specimens available from the patients participating in this erlotinib study.

Specific Aim 2.2. To investigate whether the resistance to erlotinib is mediated by the activation of type I insulin-like growth factor receptor (IGF-1R) signaling pathway

(PI: Ho-Young Lee, Ph.D.)

Because overexpression of the epidermal growth factor receptor (EGFR) has been observed in a number of common solid tumors, including 40–80% of NSCLC, EGFR has been extensively studied (Jemal et al., 2003). Therefore, one effective therapeutic strategy may be the use of agents targeting the EGFR pathway. However, negative results from several large-scale phase III clinical trials in lung cancer have been reported (Giaccone et al., 2002; Johnson et al., 2002), indicating the need for understanding the mechanisms that induce the resistance to EGFR inhibitors. Accumulating evidence has implicated insulin like growth factor-I receptor (IGF-1R) pathways in resistance to chemotherapy, radiation therapy, and molecularly targeted agents (Kulik et al., 1997; Lin et al., 1999; DiGiovanni et al., 2000; Porras et al., 1998; Toker et al., 2000). Our objectives are to investigate whether IGFR and downstream signaling mediators, such as PI3K/Akt and MAPK, are involved in the resistance to anti-EGFR therapies in NSCLC.

Objective 1: Determine whether inhibition of the IGF-1R–mediated signaling pathway augments the antiproliferative effects of erlotinib on NSCLC

cells *in vitro,* and investigate the mechanism by which erlotinib leads NSCLC cells to activate the IGF-1R signaling pathway.

Objective 2: **Determine whether inhibition of the IGF-1R–mediated signaling pathway augments effects of erlotinib on the growth of human NSCLC xenograft tumors established in nude mice.**

Update

In the past year, we have investigated whether inhibition of the IGF-1R-mediated signaling pathway augments the antiproliferative effects of erlotinib on NSCLC cells *in vitro.* We also studied the mechanism through which erlotinib leads to activation of the IGF-1R signaling pathway in NSCLC cells.

We found that the EGFR tyrosine kinase inhbitor (TKI) erlotinib induced differential apoptotic responses in NSCLC cells and that IGF-1R activation interferes with the antitumor activity of erlotinib. Treatment with erlotinib increased the levels of EGFR:IGF-1R heterodimer localized on cell membrane, activated IGF-1R and its downstream signaling mediators, and stimulated mammalian target of rapamycin (mTOR)-mediated *de novo* protein synthesis of EGFR and survivin in NSCLC cells. Accordingly, inhibition of IGF-1R activation, suppression of mTOR-mediated protein synthesis, or knock-down of survivin expression abolished resistance to erlotinib and induced apoptosis in NSCLC cells *in vitro*.

To determine whether the inhibition of IGF-1R signaling can enhance the antitumor activities of erlotinib *in vivo*, we tested the effects of erlotinib, Ad-dnIGF-1R (adenovirus-dominant negative IGF-1R), and their combination on the growth of H1299 NSCLC xenograft tumors established in athymic nude mice. The mice treated with erlotinib plus Ad-dnIGF-1R showed synergistically reduced tumor growth compared with the control mice or the mice treated with erlotinib or Ad-dnIGF-1R alone. At the end of the study, the mean tumor volume in combined treatment group was 23% ($P < 0.001$) of the mean volume in the control group. Thus, the combination of erlotinib and Ad-dnIGF-1R enhanced the antitumor effects on the growth of NSCLC cells *in vivo.*

We then determined the effects of erlotinib, Ad-dnIGF-1R, and their combination on the activation of the IGF-1R and EGFR, the expression of survivin and EGFR, and the induction of apoptosis *in vivo*. We found that erlotinib treatment induced marked increases in the levels of pIGF-1R and survivin, all of which were effectively blocked by Ad-dnIGF-1R. Combined treatment with erlotinib and Ad-dnIGF-1R also increased the levels of Ac-caspase-3, which is confirmed by the immunohistochemical staining of the H1299 xenograft tumor tissues. Together, these findings suggested that the combined treatment with erlotinib and Ad-dnIGF-1R exert enhanced *in vivo* antitumor activities by decreased expression of survivin and EGFR and induction of apoptosis.

These findings are detailed in an article published in *Cancer Research* (Morgillo et al., 2006) and two meeting abstracts (see the Appendix A - Publications).

Objective 3: Investigate whether IGF-1R activity influences the therapeutic activity of erlotinib in patients with NSCLC.

Experiments associated with this Aim are expected to begin this year with the receipt of patient specimens from the BATTLE clinical trials.

Key Research Accomplishments:

- Erlotinib enhances synthesis of survivin by inducing IGFR/EGFR heterodimer cell membrane localization, which in turn counteracts the antitumor action of the drug.
- The combination of erlotinib and Ad-dnIGF-1R synergistically inhibits the growth of tumors in xenograft mouse models.

Reportable outcomes:

Articles published in Peer-Reviewed Journals

- Morgillo F, Woo JK, Kim ES, Hong WK, Lee H-Y. Heterodimerization of IGFR/EGFR and Induction of Survivin Expression Counteracts the Antitumor Action of Erlotinib. *Cancer Res*, 66(20):10100-11, 2006.

Abstracts

- Lee H-Y Morgillo F, Hong WK. Insulin-like growth factor-1 receptor/epidermal growth factor receptor (EGFR) heterodimerization and resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non–small cell lung cancer. American Society of Clinical Oncology Annual Meeting, Abstract #OR02-1, 2006.
- Morgillo F, Woo JK, Hong WK, Ciardiello F, Lee H-Y. Induction of survivin expression *via* activation of insulin-like growth factor-1 receptor/epidermal growth factor receptor heterodimer: A novel resistance mechanism of EGFR tyrosine kinase inhibitors in non–small cell lung cancer. The 5th Annual AACR International Conference Frontiers in Cancer Prevention Research, Abstract #428, 2006.

Conclusion:

Our findings indicate the need for integration of IGF-1R-targeted agents to treatment regimens using EGFR TKIs for patients with lung cancer.

Specific Aim 2.3. To investigate the molecular mechanisms of resistance to and biomarkers of the biologic activity of inhibitors of the VEGF pathway

(PI: John Heymach, M.D., Ph.D.)

The primary goals of this Aim were to develop biomarkers for the activity of VEGF inhibitors and investigate potential markers of therapeutic resistance. Substantial progress has been made towards these goals. The focus of our effort thus far has been in the development of our methodologies for the blood based biomarkers (objectives 2 and 3), in large part because of specimen availability. Notable advances over the past year, detailed below, include the following: 1) Development of techniques for assessing VEGF receptor-bearing monocytes by 7-color flow cytometry, and initial validation using clinical samples demonstrating that these populations are specifically modulated by VEGF inhibitors as compared to non-VEGF receptor bearing populations; 2) Development of methodologies for assessing different circulating

endothelial cell populations, and 3) Application of plasma profiling of angiogenic factors to identify potential mechanisms of resistance to VEGF inhibitors. In particular, we have developed a profile of cytokine and angiogenic factors and, in our initial application of this platform, we have identified the HGF/MET axis as potentially being involved in resistance to ZD6474.

The objectives of this aim have not been modified since the project began. Progress on these objectives is detailed below.

Objective 1: Quantitatively assess VEGFR phosphorylation, downstream signaling, and biomarkers of angiogenesis in pre- and post-treatment tumor biopsy samples.

Update

This objective requires quantitative analysis of tumor specimens pre- and post-treatment from patients enrolled in the BATTLE protocol using laser scanning cytometry to detect angiogenesis, endothelial apoptosis, and VEGF receptor phosphorylation. We have further refined our methods for this analysis using human xenograft tumors (A549) in mice and confirmed that the VEGFR inhibitor ZD6474 inhibits angiogenesis and increases endothelial apoptosis as compared to untreated controls of the EGFR inhibitor erlotinib. The analysis of the available clinical tumor specimens from patients treated in the BATTLE protocols will be done in a series of batch analyses, which we anticipate will occur in this upcoming year.

Objective 2: Investigate the utility of circulating endothelial cells (CECs), monocytes, and other cells in peripheral blood as biomarkers for antiangiogenic activity and inhibition of the VEGF pathway.

Update

A major focus of our efforts has been to develop methods for identifying specific populations of circulating endothelial cells (CECs), circulating endothelial progenitors (CEPs), and monocytes, particularly those bearing VEGF receptor 1 (VEGFR1+ monocytes), which may serve as potential biomarkers for VEGF inhibitors. Previously, using our earlier 4-color flow cytometry method, we established that in patients treated with the VEGFR inhibitor sunitinib, both CECs and monocytes were modulated during treatment. These changes differed in patients who had progressive disease and clinical benefit, suggesting that these may be useful biomarkers for VEGFR inhibitors (Norden-Zfoni et al., 2007).

Over the past year, we have significantly enhanced our ability to assess both CECs and different monocyte populations-including those specifically bearing VEGF receptors- through the development of two 7-color flow cytometry panels.

CECs/CEPs: We are now capable of evaluating multiple relevant phenotypic definitions of CECs/CEPs simulataneously through an expansion of our methodology in the following way. After immuno-blocking, normal PBMCs were stained and analyzed on a FACSCanto (BD Biosciences, Franklin Lakes, NJ) using a 7-color antibody panel: a cell permeable nuclear stain (Syto16; Molecular Probes, Carlsbad, CA), three different endothelial markers (PE-VEGFR2, PerCP-Cy5-CD146, PerCP-Cy7-CD31), two progenitor markers (PETR-CD34, APC-CD133) and a marker of hematopoietic cells for exclusion (APC-Cy7-CD450). A supplemental 5-color panel including CD3, CD19 monoclonal antibodies was used to more specifically identify and

exclude lymphocytes. Fluorescence-labeled isotype-matched nonspecific IgG_1 antibodies were used as negative controls. HUVEC (endothelial cells), CD133+ WERI cells, and CD34+ KG1A cells were mixed and spiked into the PBMC pool as positive controls. Viability was assessed by apoptosis/nucleic-staining of Syto16; and only viable, low to medium side scatter lineage-negative singlets were analyzed. Inter-assay variability was evaluated by 20 independent experiments and the coefficient of variance (CV%) was found to be under 15% for the relevant CEC populations. Because there are several different proposed subpopulations for CECs and CEPs that have emerged in the past year (Duda et al., 2006; Mancuso et al., 2006), we include in our analysis the phenotypic definitions previously evaluated by our group (Norden-Zfoni et al., 2007) as well as those proposed by other investigators. These include multiple subpopulations of CECs (CD45-/CD133-/CD146+, CD45-/CD133-/CD146+/CD31+, CD45-/CD133-/CD31+, CD45-/CD133-/CD34+, CD45-/CD133-/VEGFR2+), and CEPs (CD45-/CD133+/VEGFR2+, CD45-/CD133+/VEGFR2-/CD34+, CD45-/CD133+/CD34+, CD45-/CD133+/CD146+, CD45-/CD133+/CD31+).

Monocytes: In early efforts to identify potential cellular biomarkers for the VEGF pathway, we screened peripheral blood for VEGFRs and identified monocytes as a major VEGFR1–expressing and VEGF-binding population (Norden-Zfoni et al., 2007). This discovery raised the possibility that the VEGFR1+ monocytes may serve as a marker for VEGFR inhibitors. To explore this possibility, and further characterize different populations of monocytes that express therapeutically relevant RTKs, we have now a 7-color flow cytometry panel that includes, in addition to VEGFR1, the monocyte marker CD14; the nuclear stain syto16; the receptor tyrosine kinase receptors PDGFR-β, cKit, and Tie2, which are all relevant therapeutic targets; CD34, a progenitor marker; CXCR-4, a marker of hemangiocyte progenitors, a population of VEGFR1 bearing monocytes recently found to play an important role in promoting angiogenesis (Jin et al., 2006). Using multi-colored flow cytometry, CD14+VEGFR-1+/- monocytes are identified, and cell subpopulations including PDGFR-β vs. cKit-expressing and Tie2 vs. CXCR-4-expressing CD14+ VEGFR-1+/- have been quantified in a set of blood from normal controls.

In the upcoming year, these methods will be used to assess samples that are currently being collected and batched from the BATTLE protocol. As an initial step in investigating the utility of these different subpopulations as biomarkers for VEGF inhibitors, we applied these methods to specimens from several clinical trials of VEGF inhibitors including those from a trial of ZD6474 and another from the VEGF inhibitor AZD2171 (these trials are not part of the current BATTLE studies but we have applied the methods developed for evaluation of the BATTLE specimens as an initial means towards validating these markers). In patients treated with AZD2171, we found VEGFR1+CD14+ monocytes were decreased by approximately 80% during the first two cycles of treatment, while the general monocytic population and other PBMCs, such as lymphocytes, did not change appreciably during treatment (data not shown). This provides support for our hypothesis that VEGFR1+ monocytes may serve as a specific, blood-based marker for VEGFR inhibitors.

Objective 3: Systematically examine changes in the plasma and serum angiogenic profiles consisting of a panel of proangiogenic cytokines, targeted receptors, and potential biomarkers of endothelial damage.

Update

Based on our prior preclinical studies of ZD6474 in murine models, and our clinical studies of biomarkers for other VEGF inhibitors, we have tested and developed a profile of 35 different cytokines and angiogenic factors (CAF profile) using multiplexed beads assays, including

VEGF, bFGF, EGF, hepatocyte growth factor (HGF), E-selectin, ICAM-1, MMP-9, and a broad set of interleukins and chemokines. This method permits us to conduct analyses using less than 200 μl of total plasma. This profile will be applied during the upcoming year to batched samples collected from the BATTLE protocols. To initially test this CAF profile and identify potential markers of resistance to ZD6474, we applied the profile to samples from a recently completed randomized phase II clinical trial of ZD6474, alone or in combination with chemotherapy

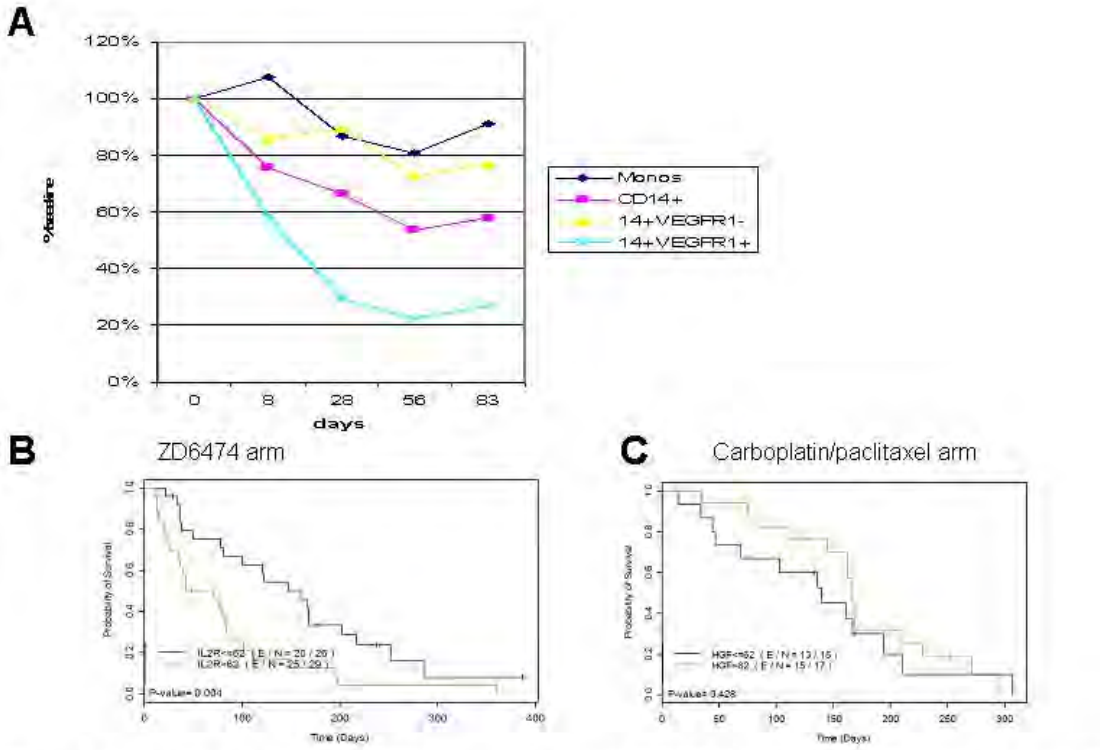


Figure 7. A. Specific targeting of VEGFR1+ monocytes by the VEGFR inhibitor AZD2171. Using multicolor flow cytometry, subpopulations of monocytes were assessed prior to treatment (day 0) and during treatment. VEGFR1+CD14+ monocytes were inhibited by up to 80% during treatment; the overall monocyte population, by contrast, was inhibited by less than 20%. Monocytes coexpressing VEGFR1 and PDGFR were inhibited by approximately 90% at day 56 (data not shown). Predictive plasma markers of benefit from patients treated with ZD6474 in a randomized phase II trial (Heymach et al, Proc ASCO 2007; Hanrahan et al, Proc ASCO 2007). (B) Patients with high plasma HGF treated with ZD6474 demonstrated a trend towards shorter progression free survival than those with low HGF; (p=0.06) by contrast, a trend towards improved PFS was seen in patients with high plasma HGF in patients treated with carboplatin and paclitaxel (C) (p value for interaction p=0.04).

(carboplatin and paclitaxel) vs. chemotherapy alone. Plasma samples were collected at baseline (N=123 patients), as well as day 8 and at the end of cycles 1 and 2. Controlling for covariates, tests for treatment by factor interactions, which assess whether the treatment effect was different in patients with low and high marker levels, were significant for baseline HGF (p=0.04) and IL-2R (p=0.008). Both HGF and IL-2R were predictive of responsiveness to ZD6474 but not the chemotherapy: low levels were associated with prolonged PFS in the ZD6474 arm, but were not associated with differences in the CP or Z+CP arms (Figure 7B and C).

Key Research Accomplishments:

- Assessed VEGFR phosphorylation and angiogenesis biomarkers (microvessel density by CD31 staining, HIF-1alpha activation) in human xenografts (A549, HCC827) treated with vehicle, erlotinib, and VEGF inhibitors (i.e., ZD6474) to confirm angiogenesis inhibition and signaling blockade using laser scanning cytometry.

- Developed 7-color flow cytometry method for assessing different populations of circulating endothelial cells (CECs).
- Identified novel subsets of VEGFR-1 bearing monocytes and developed methodology for quantifying these subsets in clinical specimens using 7 color flow cytometry.
- Applied these new CEC and monocyte evaluations to specimens from clinical trials of VEGFR inhibitors to demonstrate subpopulations that are specifically targeted.
- Developed plasma cytokine/angiogenic factor (CAF) profile of 35 different factors including angiogenic factors, EGF family members, interleukins, chemokines, markers of hypoxia, and endothelial damage.
- Measured CAF profile using multiplexed bead arrays in specimens from clinical trials.
- Identified HGF/MET axis as potential mediator of resistance to ZD6474.

Reportable Outcomes:

Abstract
Heymach JV, Hanrahan EO, Lin HY, Du DZ, Yan S, Kim ES, Lee JJ, Ryan AJ, Tran HT, Johnson BE. Correlative analyses of plasma cytokine / angiogenic factor (C/AF) profile, gender and outcome in a randomized, three-arm, phase II trial of 1st-line vandetanib (VAN) and / or carboplatin plus paclitaxel (CP) for advanced non small cell lung cancer (NSCLC). American Society of Clinical Oncology Annual Meeting, Abstract, June 2007.

Conclusion:
This data generated during the first year of our study supports our hypothesis that blood-based assays may provide markers of activity as well as potential predictive markers for selecting patients. In the upcoming year, we will be applying these methods to the batched samples collected to date from the BATTLE protocol to further investigate these markers, particularly for the VEGF inhibitors sorafenib and ZD6474.

Specific Aim 2.4. To investigate the molecular mechanisms of the effects of the combination of bexarotene and erlotinib on NSCLC cells

(PI: Reuben Lotan, Ph.D.)

Objective 1: To determine by immunohistochemical analysis the expression of nuclear receptors (retinoic acid receptors [RAR]-α, -β, and -γ; RXR-α, -β, and -γ; and PPAR-γ1 and PPAR-γ2) and cyclin D1 in NSCLC specimens obtained from patients to be enrolled in the BATTLE umbrella trial and from patients whose cancer progresses on treatment.

Update

We have made some initial progress on the analysis of nuclear retinoic acid receptors using tissue specimens collected from NSCLC patients.

Initially, we performed IHC analysis of more than 100 cases included in tissue microarrays prepared by the Biomarker Core (Dr. Ignacio Wistuba) for the expression of 6 nuclear retinoid receptors including RARα, RARβ, RARγ, RXRα, RXRβ, and RXRγ. We are currently in the process of quantifying these IHC staining data and will have more definitive results in the next report.

In addition, as a part of the effort in biomarker analysis for stratifying the BATTLE patients into the treatment arms, RXRs and cyclin D1 were analyzed in the enrolled 33 patients by the BATTLE Biomarker Core (Table 3).

Table 3. Summary of biomarker results in 33 NSCLC cases. Expression was determined by IHC analysis.

Biomarker Group	Positive Cases
EGFR markers	5 (15%)
EGFR mutation (exons 18-21)	4 (12%)
EGFR FISH increased copy number	18 (55%)
K-Ras/B-Raf	5 (15%)
KRAS mutation (codons 12, 13 and 61)	4 (12%)
BRAF mutation (exons 11 and 15)	1 (3%)
Angiogenesis	28 (85%)
VEGFR expression (score >100)	
VEGFR-2 expression (score >100)	
RXRs/CyclinD1	30 (91%)
RXR α nuclear expression (score >30)	26 (79%)
RXR α cytoplasmic expression (score >200)	0
RXR β cytoplasmic expression (score >200)	4 (12%)
RXR β membrane expression (score >200)	0
RXR γ cytoplasmic expression (score >200)	2 (6%)
Cyclin D1 Expression (score >10%)	14 (42%)
Cyclin D1 FISH amplification	0

Among 30 positive cases in the expression of RXRs/cyclinD1 as determined by IHC analysis, RXRα was detected in the nuclei of 79% of the cases (26 of 30), RXRβ in the cytoplasm of 12% of the cases (4 of 30), RXRγ in the cytoplasm of 6% of the cases (2 of 30), and cyclin D1 in 42% (14 of 30). For details, please refer to the report of Biomarker Core below.

Objective 2: Examine the effects of bexarotene, erlotinib, and rosiglitazone alone and in combination on the growth and apoptosis of NSCLC cells, cyclin D1 and PPAR-γ levels, and gene expression profiles.

Update

Progress was also made on this objective. Specifically, we have examined the effects of the RXR selective retinoid bexarotene on lung cancer cells in culture. We found that at concentrations up to 1 μM bexarotene as a single agent, which is achievable in patients, there was only a low growth inhibition. This warrants our plan to combine this agent with erlotinib and rosiglitazone, a PPAR-γ thiazolidinedione agonist, which we will pursue in the coming year.

Objective 3: Determine whether RXRs, EGFR, and PPAR-γ are required to mediate the effects of bexarotene, erlotinib, and rosiglitazone, respectively, on

cell growth control and apoptosis, and examine the functional significance of changes in gene expression induced by receptor agonists used singly or in combinations.

Objective 4: Evaluate the growth inhibitory effects and mechanisms of action of novel RXR ligands AGN194204 and 9cUAB30 alone or combined with erlotinib and rosiglitazone on NSCLC cells.

Update

Research for these aims is scheduled to begin this year.

Key Research Accomplishments:

- Optimized the conditions of immunohistochemical analysis and quantitation of the nuclear retinoid receptors.

Reportable Outcomes:
None

Conclusions:
We conclude that the majority of NSCLC cases express nuclear RXRα but expression of the two other RXR receptors (RXRβ and RXRγ) is very limited. Also, the use of bexarotene as a single agent in NSCLC appears to be limited and combination therapy will be tested.

Specific Aim 3: To identify biomarkers as novel predictors of clinical end points and potential therapeutic targets

(PI: Li Mao, M.D.)

Objective 1: Identify molecular features in tumor tissues that correlate with patients' responses to individual regimens used in the clinical trials of the proposed program.

Objective 2: Determine the effect of targeted agents in tumor tissues, and identify novel molecular mechanisms of tumor response or progression.

Objective 3: Identify serum biomarkers as surrogate markers to predict patients' responses to individual treatment regimens.

Update

We have been actively participating in planning and initiation of the clinical trial. The clinical trial is moving smoothly with approximately 85% of the biopsies available for experiments proposed in this aim. In addition to serum samples collected from the clinical trial, we have suggested to include the collection of platelets for measurement of certain growth factors such as VEGF because the platelets may serve as scavengers to transport these growth factors to tumors and play an important role in tumor progression. Because this aim is heavily dependent on samples and clinical data from the clinical trials, the performance of the aim is planned to begin this

coming year. Currently, we are evaluating the suitability of the clinical samples collected from the trials for the various experiments proposed in this aim.

Key Research Accomplishments:
None

Reportable Outcomes:
None

Conclusions and Future Plans:
In the next year, we will perform whole genome expression analysis in the first 50 tumor tissues to identify potential signatures associate with biomarker status measured in the Pathology Core. Potential signatures with strong association with clinical parameters, particularly PFS, will be emphasized. We will also construct the first panel of reverse-phase protein microarrays from the tissues and the corresponding serum. The first priority of the tissue use will be given to gene expression microarrays, followed by reverse-phase protein microarrays. Remaining tissues will be used for protein marker discovery using antibody arrays and 2-DE strategies as outlined in our research proposal. Selected candidate genes/proteins will be validated using quantitative RT-PCR and reverse-phase protein microarrays.

Specific Aim 4: To explore new preclinical combinations and their mechanisms of action by targeting mTOR signaling and develop phase I trials to test these combinations.

(PI and Co-PIs: Fadlo Khuri, M.D., Shi-Yong Sun, Ph.D., Haian Fu, Ph.D.)

The overall objective of Aim 4 is to study the efficacy of mTOR inhibitor combination therapies that co-target mTOR and PI3K/Akt signaling. Our progress is summarized below.

Objective 1: To study the efficacy of mTOR inhibitor combination therapies that co-target mTOR and PI3K/Akt signaling.

Update

One proposed study is to examine the effects of an mTOR inhibitor such as rapamycin combined with Tarceva (erlotinib) on the growth of NSCLC cells. Thus, we did the experiment by examining the effects of the combination on the growth of human NSCLC cell lines in a 3-day monolayer culture and in a 9-day colony formation assay. In the 3-day assay, the combination of rapamycin and Tarceva exerted synergistic inhibitory effects on the growth of several NSCLC cell lines according to the calculation using the CalcuSyn software (CI <1) (Chou et al., 1984). The synergistic effects of the combination in multiple NSCLC cell lines were further confirmed in a long-term (9-day) colony formation assay. As presented in Figure 8, the combinations of rapamycin (ranging from 0.25 nM to 1 nM) with either 2.5 μM or 5 μM Tarceva were much more potent than each single agent in inhibiting the formation and/or growth of colonies; these effects appeared to be more than additive effects. Thus, these data clearly indicate that co-targeting mTOR and EGFR/PI3K/Akt signaling exerts synergistic effect on inhibiting the growth of human NSCLC cells, thus providing a strong scientific rationale for testing the combination of an mTOR inhibitor with Tarceva in animal models and in human clinical trials as proposed in this aim.

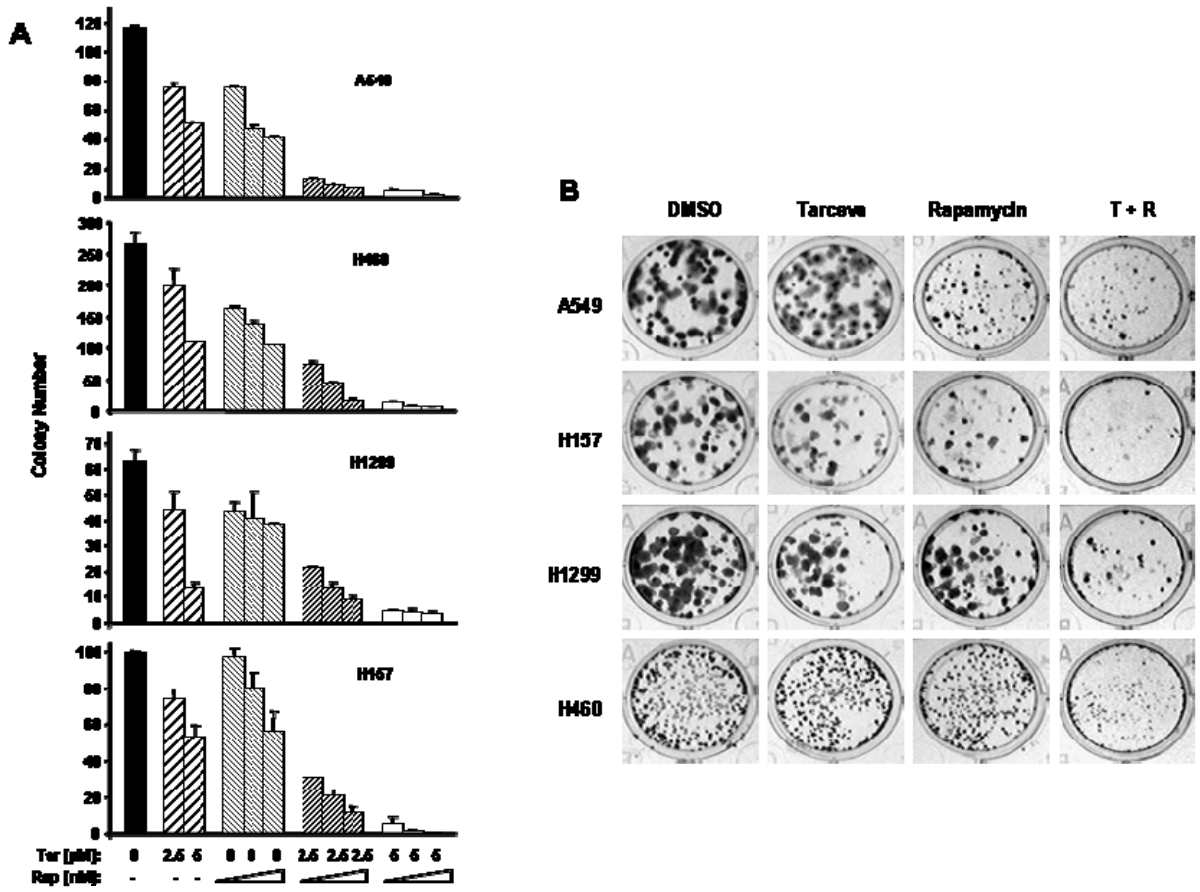


Figure 8. Effects of rapamycin combined with Tarceva on the growth of human NSCLC cells evaluated in a 9-day colony formation assay. *A*, Quantitation from duplicate assays. Tar, Tarceva; Rap, rapamycin. The concentrations of rapamycin were 0.25, 0.5 and 5 nM. *B*, Representative pictures of the assays presented in *A*. R + T, rapamycin plus Tarceva.

Objective 2: To examine whether rapamycin-induced Akt activation suppresses ASK1-mediated apoptosis and leads to decreased therapeutic efficacy.

Update

We proposed to test the functional interaction of Akt and apoptosis signal-regulating kinase 1 (ASK1) and the effect of Akt/ASK1 modulators, such as Hsp90, on ASK1-mediated apoptosis in order to enhance the efficacy of rapamycin based therapy. During the first year of the grant, we primarily focused on developing reagents and on establishing experimental models to ensure the success and efficient execution of the proposed studies. To this end, we have conclusively demonstrated the expression of ASK1 protein in lung cancer cells. Upon activation of the Akt pathway, we showed that Ser83 of ASK1 became phosphorylated. In addition, we have identified a novel Akt-regulated phosphorylation event on ASK1 either directly or indirectly through phosphorylation of ASK1-Ser967. Ser967, previously discovered in our lab, controls the pro-apoptotic function of ASK1. Phosphorylation of ASK1-Ser967 induced the binding of 14-3-3 proteins to ASK1, leading to suppression of ASK1-mediated apoptosis. Thus, activated Akt may act through two different mechanisms to inhibit ASK1 by either phosphorylating Ser83 or

Ser967. These findings have set a stage for the proposed experiments to link rapamycin-induced Akt activation with the action of ASK1.

One of the regulators of Akt and ASK1 is Hsp90. We have generated preliminary evidence that ASK1 in lung cancer cells is associated with Hsp90. To gain initial insight into therapeutic implications of this finding, we examined the effect of Hsp90 inhibitor, 17-AAG, on Akt activity in lung cancer cells using a demonstrated Akt substrate (PRAS40) phosphorylation as readout. Indeed, the treatment of cancer cells with 17-AAG induced the gradual dephosphorylation of PRAS40. This effect is dose- and time-dependent. We will continue this line of research to establish the inhibition of Hsp90 and the activation of state of ASK1 in lung cancer cells.

Objective 3: To conduct two phase I clinical trials to test the efficacy of the combination of an mTOR inhibitor with an Akt or an EGFR inhibitor in advanced NSCLC patients resistant to the front and second line therapy, and assess the modulation of targeted biomarkers from tumor tissues before and after the treatment.

Update

This study is under active development, and discussions have been held with both Caelyx Pharmaceuticals and two potential sponsors in Wyeth and Novartis for supply of Akt inhibitor, perifosine and mTOR inhibitors, Temsirolimus/CCI-779 or Everolimus/RAD-001, respectively. We have received assurances from Caelyx for supply of perifosine and are in the process of negotiating with the other two companies for supply of one of the two mTOR inhibitors. We are concentrating on opening this trial of perifosine plus mTOR inhibitor, and will then begin the trial of erlotinib plus RAD001.

The trials are planned to start in year 3 of the BATTLE grant.

Key Research Accomplishments:

- Demonstrated the synergistic effect of the combination of rapamycin and Tarceva on the growth of human NSCLC cells based on the *in vitro* cell culture results.
- Detected ASK1 in lung cancer cells and linked Akt activation to phosphorylation of ASK1 at Ser83.
- Discovered a new mechanism by which Akt activates ASK1 through phosphorylation of Ser967 and induced 14-3-3 protein complex formation of 14-3-3 and ASK1.
- Demonstrated the effect of Hsp90 inhibitor on inhibiting the phosphorylation of a new Akt substrate, PRAS40.

Reportable Outcomes:
None

Conclusions:
We conclude that the combination of rapamycin and Tarceva synergistically inhibited the growth of human NSCLC cells, thus supporting our hypothesis that co-targeting PI3K/Akt activation and suppression of mTOR signaling will result in enhanced anticancer activity; our study also reveals the expression of ASK1 in lung cancer cells and the action of Hsp90 inhibitor in blocking Akt phosphorylation. These data support our proposal to examine the role of ASK1 signaling pathway in mediating rapamycin-induced therapeutic effect.

Biostatistics and Data Management Core:

(Core Director: J Jack Lee, Ph.D.)

In close collaboration with the Biomarker Core, the clinical research team, and each of the basic science research components, the Biostatistics and Data Management Core (BDMC) for the Department of Defense (DoD) BATTLE lung cancer research program will be a comprehensive, multi-lateral resource for designing clinical and basic science experiments; developing and applying innovative statistical methodology, data acquisition and management, and statistical analysis; and publishing translational research generated by this research proposal.

The BDMC will incorporate sound experimental design principles within each BATTLE program project that will increase the clarity and enhance the interpretability of study results. Each project will be provided with tailored analyses, accompanied by novel statistical development as necessary, to reveal apparent and hidden relationships among data. The BDMC will provide expertise in the design of an integrated data management system to facilitate communication among all projects and cores. This process includes prospective data collection, data quality control, data security, and assurance of patient confidentiality. The BDMC will collaborate with all project investigators to facilitate the timely publication of data collected under the BATTLE research program.

The main **objectives** of the BDMC are as follows:

1) Develop and implement a novel adaptive randomization scheme for assigning patients into the treatment arms with the highest probability of success.
2) Provide the statistical design, sample size, and power calculations for each project.
3) Develop a secure, internet-driven, web-based database network between UTMDACC and other research centers, including Emory University and the Dana-Farber Cancer Institute, which integrates the clinical data generated by the five proposed clinical trials and relating basic science research efforts of the BATTLE research project.
4) Develop a comprehensive, web-based database management system for tissue specimen tracking and distribution and for a central repository of all biomarker data.
5) Provide all statistical data analyses, including descriptive analysis, hypothesis testing, estimation, and modeling of prospectively generated data.
6) Provide prospective collection, entry, quality control, and integration of data for the basic science, pre-clinical, and clinical studies in the BATTLE grant.
7) Provide study monitoring and conduct that ensures patient safety by timely reporting of toxicity and interim analysis results to various institutional review boards (IRBs), the UTMDACC data monitoring committee, the DoD, and other regulatory agencies.
8) Generate statistical reports for all projects.
9) Collaborate with all project investigators and assist them in publishing scientific results.
10) Develop and adapt innovative statistical methods pertinent to biomarker-integrated translational lung cancer studies.

Update:

In the first funding year, the BDMC worked with all project investigators in providing biostatistics and data management support, and summarized the details below.

(A) Biostatistics

We have developed a novel study design incorporating hierarchical Bayes model and adaptive randomization to adaptively randomize more patients into more effective treatments based on each patient's biomarker profile. Extensive simulations have been conducted to choose the design parameters such that the design has desirable statistical properties to ensure: 1) high probability of identifying effective treatments; 2) high probability of suspending/terminating the ineffective treatments; and 3) randomizing more patients into the effective treatments based on each patient's biomarker profile.

We have worked with clinical investigators in providing the biostatistical support in the development, revision, and approval of the five protocols (one umbrella protocol and four treatment protocols). We have provided statistical reports in our monthly BATTLE meetings to share an update of the accrual, randomization, demographic data, etc, with all investigators.

(B) Data Management

We have made extensive efforts in BATTLE Database development. Database tasks are listed below:

1) Developed user security to allow read/write or read only access to specific parts of the BATTLE database application.
2) Applied a consistent user interface throughout the application.
3) Designed a user-friendly screen/CRF navigation on the left side of application.
4) Adhered to HIPAA regulations for patient confidentiality.
5) Supported multiple consents as required by the BATTLE protocols.
6) Presented Inclusion and Exclusion criteria questions in an easy to use, consistent format.
7) Biomarker results are displayed when Biomarker information is entered as well as Marker Group Assignment for Randomization.
8) Randomization screen displays Consent information to help maintain an easy work flow.
9) Randomization is disabled until a patient signs the consent, and biomarker entry and eligibility information are entered.
10) Supported two types of randomization, "equal randomization" until we have enough marker groups and treatments followed by "adaptive randomization." An ability to randomize when biomarker information is not available and without affecting the adaptive randomization also exists.
11) The Randomization screen also has consent for treatment information.
12) Medical History has a large area for all medical history related information. Various grids are displayed and can be easily expanded to allow for as many entries as needed by the nurse.
13) Physical Exam, Lab Tests, Study Drug Compliance Calculation, On Study EKGs and other CRF's are cycle based. These allow for similar screens to be entered for every cycle. In special cases, information can also be entered between cycles if needed.
14) Lab Tests screen supports Urinary Analysis Not Done to avoid data entry confusion.
15) Eight-Week response saves the response information so that Adaptive Randomization can use this information to help ensure the patient gets assigned to the most effective treatment.
16) Tumor Measurement entry allows for as many lesions as needed.
17) Support for general comments can be entered as needed for every patient.

Database programming efforts are described as follows.

1) The C# (C Sharp) ASP 2.0 Application is running on an IIS web server using SSL for additional security and encryption. Microsoft SQL Server 2000 is used to store the data as the User/Login information.

2) The BATTLE system uses Attributes to store study related information. These attributes have unique ID's and are grouped by form so that they are easily retrieved when a specific form is brought up for a specific patient. Having these attributes available allows for quick form design, form enhancement and organized data retrieval.

3) All CRF information is stored in Attributes in the DMI_EAV_Production Database based on Form Name, Patient ID Cycle, Attribute ID and Event ID. This allows for a wide variety of combinations to meet the future growth and virtually any need that may come up in the future for the BATTLE project.

4) There are two Randomization tables stored on the SQL server that allow for both Adaptive and Non-Adaptive (Equal) Randomization storage. The Non-Adaptive Randomization table allows us to randomize patients equally in the first phase of the trial conduct and for those who have insufficient biomarker information for applying adaptive randomization.

5) Randomization routines have been written in 'R' code, which is a high-level statistical software. The BATTLE database system calls a Web Service to run these randomization routines. The parameters, matrixes and results of these randomizations are stored in randomization tables in the DMI_EAV_Production database.

6) Specimen and Tracking data is stored in the BATTLE 2005-0823 database. This database is much more specialized and specifically tailored to the needs of BATTLE's current and future tracking needs. Since data for tracking is usually queried using large amounts of data, these tables are designed to have fast access with little to no lag.

Workflow and Database Overview and Database Screen Shots are found in Appendix B - Biostatistics Core).

This work was reported in two abstracts (Zhou et al., 2007; Liu et al., 2007) (Appendix A – Publications) and presented at two meetings as listed in the Reportable Outcomes. A manuscript is under preparation, as well.

Key Research Accomplishments:

- Developed a novel adaptive randomization design.
- Developed a secured, web-based database application to assist the study conduct.

Reportable Outcomes:

Database Resources:

A web-based database application is developed and deployed at:
https://insidebiostat/DMI_BATTLE/Common/Login.aspx

Manuscript in preparation

- Zhou X, Liu S, Lee JJ. A clinical trial design applying bayesian adaptive randomization for targeted therapy development in lung sancer - A step toward personalized medicine. Manuscript (in preparation); 2007.

Presentations

- Herbst R,Lee JJ. The BATTLE Project. Presentation to the M. D. Anderson External Advisory Board. Houston, Texas, January 2007.
- Lee JJ. Adaptive Randomization Designs for Targeted Therapy. IASLC Targeted Therapies for the Treatment of Lung Cancer. Santa Monica, California, February 2007.

Abstracts

- Zhou X, Kim ES, Herbst RS, Liu S, Wistuba II, Mao L, Lewis J, Lippman SM, Hong WK, Lee JJ. A clinical trial design applying Bayesian adaptive randomization for targeted therapy development in lung cancer - A step toward personalized medicine. Submitted to the American Society of Clinical Oncology Annual Meeting, Atlanta, Georgia. June 2007.
- Liu S, Kim ES, Zhou X, Wistuba II, Herbst RS, Lewis J, Lee JJ. An Application of Adaptive Randomization Using Hierarchical Bayes Model in a Prospective Biomarker-Based Clinical Trial. Submitted to the Joint Statistical Meeting, Salt Lake City, Utah. August 2007.

Conclusion:
In collaboration with clinical investigators, research nurses, the Biomarker Core, and basic scientists, the Biostatistics and Data Management Core has delivered the biostatistics and data management support as proposed.

Biomarker Core: Perform biomarker assessment to stratify patients into a particular arm of clinical trials and coordinate the distribution of clinical samples.

(Core Director: Ignacio Wistuba, M.D.)

The Biomarker Core, in close collaboration with the Biostatistics and Data Management Core, the Clinical Trial team, and Research Project Investigators, has played an important role in achieving the objectives proposed in the aims of the proposed BATTLE program by acquiring and processing lung cancer tissue samples and performing the biomarker analysis for the stratification of patients into the clinical trials. In addition, the Core has collected and banked tissue specimens to support mechanistic studies of response or resistance to targeted agents used in the BATTLE trials.

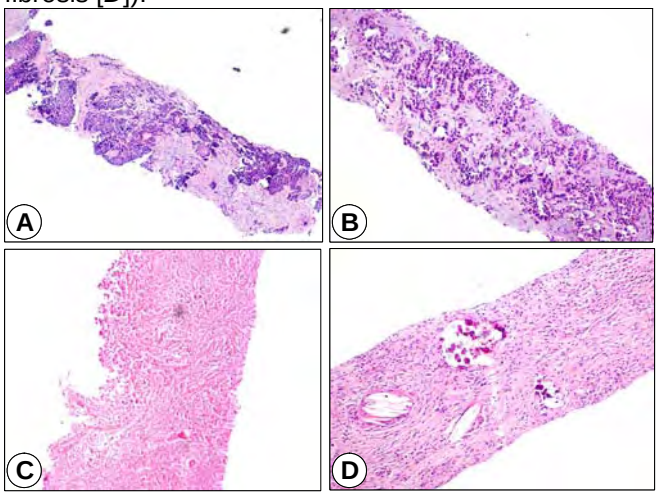
The Biomarker Core has successfully combined standard methods of histopathology processing and assessment of lung cancer tissue samples with more advanced tools of molecular and genetic biomarker analyses.

Objective 1: To acquire, bank, process, and distribute tumor and blood specimens obtained from BATTLE enrolled patients for biomarker analyses and molecular mechanistic studies of targeted agents.

Update

The Biomarker Core collected NSCLC tumor tissue core specimens from 43 patients (cases) enrolled in the BATTLE clinical trials. By March 29, 2007, NSCLC tumor core biopsies from 39 cases were processed (formalin-fixed and paraffin-embedded) and histopathologically evaluated. Of those, tumor specimens from 33 (85%) cases yielded enough tumor cells to examine and report a complete set of biomarkers as proposed (see Objective 2 - Table 2). Only in 6 (15%) patients no viable tumor cells were detected for biomarker analysis, in which the most frequent findings were necrotic tumor tissue and fibrosis. Twenty-three tissue specimens were obtained from lung sites and the rest from metastasic sites (including 10 from lymph nodes, 4 from soft tissues, and 4 from liver). NSCLC histology types included adeno-carcinoma (70%), squamous cell carcinoma (14%), and undifferentiated NSCLC (2%). At least one fresh tumor tissue core was snap frozen in each case, and those specimens were banked in our laboratory tissue bank facility. Figure 9 showed a representative of lung cancer tissue core biopsies stained by hematoxylin and eosin staining (H&E).

Figure 9. Microphotographs of lung cancer tissue core biopsy from BATTLE patients showing two specimens with adequate tissue for biomarker analysis (adenocarcinoma [*A*] and squamous cell carcinoma [*B*]) and two specimens not adequate (necrosis [*C*] and fibrosis [*D*]).



Objective 2: **To perform biomarker analyses and report results in a timely fashion for patient stratification in the BATTLE trials and molecular mechanistic studies of the targeted agents.**

Update

Tissue specimen workflow for biomarker analysis:
We have developed a system to collect and process tissue specimens, evaluate tissue quality for analysis, perform biomarker analysis, and report biomarker results into the web-based clinical trial database within 14 days.

Briefly, this process is as follows:

1. Tissue specimens cores (2 – 3 cores) are collected from Interventional Radiology by Biomarker Core's personnel.
2. Immediately, the specimens are transported to the lab for formalin fixation (1 or 2 cores) and snap freezing (1 core).
3. In 24 hours, histology sections are reviewed by a pathologist to assess tissue quality and select areas for microdissection (DNA extraction for mutation analyses) and slides for FISH (EGFR and Cyclin D1) and IHC (6 markers).
4. If the tissue specimen is suitable for biomarker analysis, FISH, IHC and mutation studies will be performed in the next 6-7 days. If the issue specimen is inadequate for biomarker

examination (including frozen specimens), this information is reported immediately to the clinical trial personnel.

5. IHC and FISH markers are performed using external and/or internal controls and mutational analyses are performed in duplicate.
6. After the biomarker analysis is completed by the Biomarker Core lab personnel, two pathologists (Drs. Ximing Tang and Ignacio Wistuba, Biomarker Core Director) will review independently the biomarker results and then report their review results by using the web-based electronic report.

Biomarker analyses:

The biomarkers that are routinely examined in the lung tumor tissue specimens are listed in Table 4. All biomarkers are examined using the formalin-fixed and paraffin-embedded tissues. DNA for mutation analyses of *EGFR*, *K-RAS* and *B-RAF* genes is extracted from microdissected tissue obtained under direct microscope observation from hematoxylin-eosin (H&E) stained tissue sections.

Table 4. Biomarkers examined in lung cancer biopsy samples and for patient stratification in clinical trials.

Molecular Pathway	Biomarkers	Type of Analysis
EGFR	*EGFR* Mutation (exons 18 to 21) *EGFR* Increased Copy Number (polysomy/amplification)	DNA sequencing DNA FISH[2]
K-Ras/B-Raf	*K-RAS* Mutation (codons 12,13, 61) *B-RAF* Mutations (exons 11 and 15)	DNA sequencing DNA sequencing
Angiogenesis	VEGF Expression VEGFR-2 Expression	Protein IHC[3] Protein IHC
RXRs/Cyclin D1	RXR α, β, γ Expression Cyclin D1 Expression Cyclin D1 Amplification	Protein IHC Protein IHC DNA FISH

[1]All. [2]FISH, Fluorescent *In Situ* Hybridization. [3]IHC, Immunohistochemistry.

Summary of biomarkers data:

As of March 29, 2007, the complete set of biomarker results was reported from 33 patients enrolled in the clinical trial. Data are summarized on Table 5, and a representative example of biomarker analysis from lung cancer tissue core specimens from a BATTLE patient was shown in Figure 10.

Table 5. Summary of biomarker results in 33 NSCLC cases.

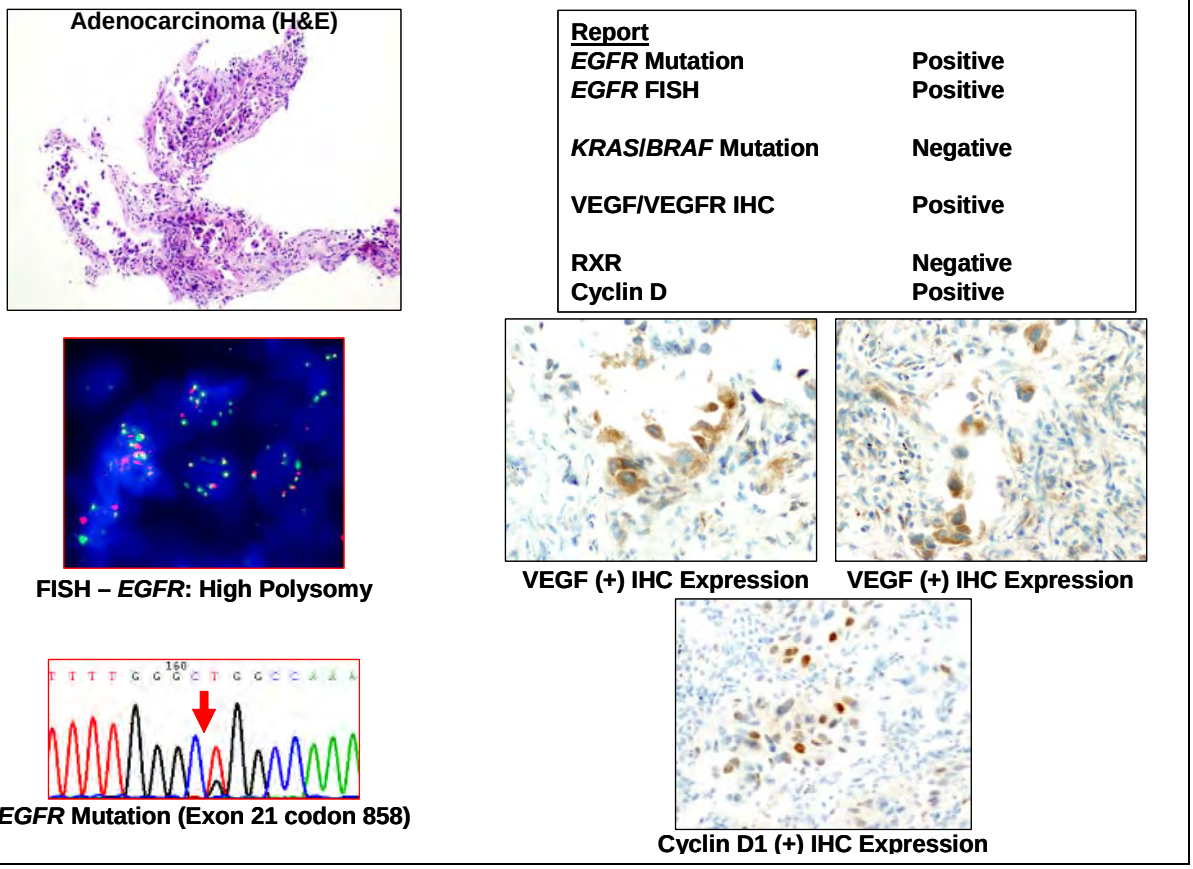
Biomarker Group	Positive Cases
EGFR markers	5 (15%)
EGFR mutation (exons 18-21)	4 (12%)
EGFR FISH increased copy number	18 (55%)
K-Ras/B-Raf	5 (15%)
KRAS mutation (codons 12, 13 and 61)	4 (12%)
BRAF mutation (exons 11 and 15)	1 (3%)
Angiogenesis	28 (85%)
VEGFR IHC expression (score >100)	
VEGFR-2 IHC expression (score >100)	

RXRs/CyclinD1	30 (91%)
RXR α nuclear IHC expression (score >30)	26 (79%)
RXR α cytoplasmic IHC expression (score >200)	0
RXR β cytoplasmic IHC expression (score >200)	4 (12%)
RXR β membrane IHC expression (score >200)	0
RXR γ cytoplasmic IHC expression (score >200)	2 (6%)
Cyclin D1 IHC Expression (score >10%)	14 (42%)
Cyclin D1 FISH amplification	0

[1]EGFR FISH Amplification 4 (12%) and High-Polysomy 15 (45%).

Formalin-fixed and paraffin-embedded histology sections and fresh frozen tissues from all cases were banked for future distribution to the BATTLE research investigators for their research activities.

Figure 10. A representative example of biomarker analysis from lung cancer tissue core specimens from a BATTLE patient.



Key Research Accomplishments:

- Established a system for tissue processing and molecular biomarker analysis of core biopsy tissue specimens from lung cancer patients in timely fashion (14 days).
- Completed biomarker analysis for clinical trial specimens on all patients within 2 weeks which is vital to the success of our clinical trials.

Reportable Outcomes:

None

Conclusions:

The Biomarker Core has successfully combined standard methods of histopathology processing and assessment of lung cancer tissue samples with more advanced tools of molecular and genetic biomarker analyses for prospectively examining molecular biomarkers for individualized targeted therapy in NSCLC patients.

KEY RESEARCH ACCOMPLISHMENTS

Specific Aim 1: To establish a clinical trial program using biomarkers to select individualized targeted therapy for patients with chemo refractory advanced NSCLC.

- Activated all five BATTLE clinical trials at M. D. Anderson Cancer Center within the first grant year.
- 63 patients registered and 38 randomized into one of the four treatment arms.
- Patient accrual and interest continue at a healthy pace.
- The success rate of the tissue acquisition and biomarker evaluation is over 85%.
- Demonstrated highly efficient collaboration of Clinical team, Biostatistics Core, and Biomarker Core.
- Developed the largest translational research program requiring core biopsy samples ever run in our department and, possibly in the country.

Specific Aim 2.1: To validate the molecular mechanisms of response and resistance to erlotinib for patients with chemo refractory NSCLC.

- Nine patients participating in the BATTLE trials have been assigned to the erlotinib trial.
- Amphiregulin (AR) is the agonist in tumor cell lines (booth lung cancer and head and neck cancer cell lines) with wild type *EGFR* that is associated with response to gefitinib and cetuximab.

Specific Aim 2.2: To investigate whether the resistance to erlotinib is mediated by the activation of type I insulin-like growth factor receptor (IGF-1R) signaling pathway.

- Erlotinib enhances synthesis of survivin by inducing IGFR/EGFR heterodimer cell membrane localization, which in turn counteracts the antitumor action of the drug.
- The combination of erlotinib and Ad-dnIGF-1R synergistically inhibits the growth of tumors in xenograft mouse models.

Specific Aim 2.3: To investigate the molecular mechanisms of resistance to and biomarkers of the biologic activity of inhibitors of the VEGF pathway.

- Assessed VEGFR phosphorylation and angiogenesis biomarkers (microvessel density by CD31 staining, HIF-1alpha activation) in human xenografts (A549, HCC827) treated with vehicle, erlotinib, and VEGF inhibitors (i.e., ZD6474) to confirm angiogenesis inhibition and signaling blockade using laser scanning cytometry.
- Developed 7-color flow cytometry method for assessing different populations of circulating endodthelial cells (CECs).
- Identified novel subsets of VEGFR-1 bearing monocytes and developed methodology for quantifying these subsets in clinical specimens using 7 color flow cytometry.
- Applied these new CEC and monocyte evaluations to specimens from clinical trials of VEGFR inhibitors to demonstrate subpopulations that are specifically targeted.
- Developed plasma cytokine/angiogenic factor (CAF) profile of 35 different factors including angiogenic factors, EGF family members, interleukins, chemokines, markers of hypoxia, and endothelial damage.
- Measured CAF profile using multiplexed bead arrays in specimens from clinical trials.

- Identified HGF/MET axis as potential mediator of resistance to ZD6474.

Specific Aim 2.4: To investigate the molecular mechanisms of the effects of the combination of bexarotene and erlotinib on NSCLC cells.

- Optimized the conditions of immunohistochemical analysis and quantitation of the nuclear retinoid receptors.

Specific Aim 3: To identify biomarkers as novel predictors of clinical end points and potential therapeutic targets.

None

Specific Aim 4: To explore new preclinical combinations and their mechanisms of action by targeting mTOR and PI3K/Akt signaling and develop phase I trials to test these combinations.

- Demonstrated the synergistic effect of the combination of rapamycin and Tarceva on the growth of human NSCLC cells based on the *in vitro* cell culture results.
- Detected ASK1 in lung cancer cells and linked Akt activation to phosphorylation of ASK1 at Ser83.
- Discovered a new mechanism by which Akt activates ASK1 through phosphorylation of Ser967 and induced 14-3-3 protein complex formation of 14-3-3 and ASK1.
- Demonstrated the effect of Hsp90 inhibitor on inhibiting the phosphorylation of a new Akt substrate, PRAS40.

Biostatistics and Data Management Core:

- Developed a novel adaptive randomization design.
- Developed a secured, web-based database application to assist the study conduct.

Biomarker Core: Perform biomarker assessment to stratify patients into a particular arm of clinical trials and Coordinate the Distribution of Clinical Samples.

- Established a system for tissue processing and molecular biomarker analysis of core biopsy tissue specimens from lung cancer patients in timely fashion (14 days).
- Completed biomarker analysis for clinical trial specimens on all patients within 2 weeks which is vital to the success of our clinical trials.

REPORTABLE OUTCOMES

Articles published in Peer-Reviewed Journals

- Morgillo F, Woo JK, Kim ES, Hong WK, Lee H-Y. Heterodimerization of IGFR/EGFR and Induction of Survivin Expression Counteract the Antitumor Action of Erlotinib. *Cancer Res*, 66(20):10100-11, 2006.

Manuscript in preparation and submitted

- Yonesaka K, Zejnullahu K, Homes AJ, Park JO, Koivunen J, Johnson BE, Jänne PA. Presence of amphiregulin autocrine-loop predicts in vitro sensitivity of *EGFR* wild type NSCLC and HNSCC cell lines to gefitinib and cetuximab. *Cancer Res* (submitted), 2007.

- Zhou X, Liu S, Lee JJ. A Clinical Trial Design Applying Bayesian Adaptive Randomization for Targeted Therapy Development in Lung Cancer - A Step Toward Personalized Medicine. Manuscript (in preparation), 2007.

Abstracts

- Heymach JV, Hanrahan EO, Lin HY, Du DZ, Yan S, Kim ES, Lee JJ, Ryan AJ, Tran HT, Johnson BE. Correlative analyses of plasma cytokine / angiogenic factor (C/AF) profile, gender and outcome in a randomized, three-arm, phase II trial of 1st-line vandetanib (VAN) and / or carboplatin plus paclitaxel (CP) for advanced non small cell lung cancer (NSCLC). American Society of Clinical Oncology Annual Meeting, Abstract, June 2007.
- Lee H-Y, Morgillo F, Hong WK. Insulin-like growth factor-1 receptor/epidermal growth factor receptor (EGFR) heterodimerization and resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non–small cell lung cancer. American Society of Clinical Oncology (ASCO) Annual Meeting, Abstract #OR02-1, 2006.
- Liu S, Kim ES, Zhou X, Wistuba II, Herbst RS, Lewis J, Lee JJ. An Application of Adaptive Randomization Using Hierarchical Bayes Model in a Prospective Biomarker-Based Clinical Trial. Submitted to the Joint Statistical Meeting, Salt Lake City, Utah, August 2007.
- Morgillo F, Woo JK, Hong WK, Ciardiello F, Lee H-Y. Induction of survivin expression *via* activation of insulin-like growth factor-1 receptor/epidermal growth factor receptor heterodimer: A novel resistance mechanism of EGFR tyrosine kinase inhibitors in non–small cell lung cancer. The 5th Annual AACR International Conference Frontiers in cancer Prevention Research, Abstract #428, 2006.
- Yonesaka K, Zejnullahu K, Homes AJ, Johnson BE, Jänne PA. Presence of amphiregulin autocrine-loop predicts in vitro sensitivity of *EGFR* wild type NSCLC and HNSCC cell lines to gefitinib and cetuximab. Submitted to The World Conference of Lung Cancer, Seoul, Korea, September 2007.
- Zhou X, Kim ES, Herbst RS, Liu S, Wistuba II, Mao L, Lewis J, Lippman SM, Hong WK, Lee JJ. A clinical trial design applying Bayesian adaptive randomization for targeted therapy development in lung cancer - A step toward personalized medicine. Submitted to American Society of Clinical Oncology (ASCO) Annual Meeting, Atlanta, Georgia, June 2007.

Presentations

- Herbst R, Lee JJ. The BATTLE Project. Presentation to the M. D. Anderson External Advisory Board. Houston, Texas, January 2007.
- Lee JJ. Adaptive Randomization Designs for Targeted Therapy. IASLC Targeted Therapies for the Treatment of Lung Cancer. Santa Monica, California, February 2007.

Database Resources:

- A web-based database application is developed and deployed at: https://insidebiostat/DMI_BATTLE/Common/Login.aspx

CONCLUSIONS and FUTURE WORK

In the first year grant period, the BATTLE program proceeded very well. The clinical trials ran even better than we expected. Six research projects have also started their *in vitro* studies and made some progress. The tissue specimens and blood samples collected from the BATTLE patients will soon be distributed to individual investigators for their projects using these samples.

This year, we have one (1) publication in *Cancer Research*, two (2) manuscripts in preparation and submission respectively, and six (6) meeting abstracts. The following conclusions for each project can be drawn:

Aim 1: The completion of the clinical trials is the key to this BATTLE research program. In the first grant year, the program has been significantly ahead of our proposed timeline. The trial accrual is reflective of the goals of the department in its completion. The design and innovative nature of the trials will keep interest high among patients who are treated at M. D. Anderson. Accrual is ongoing and will help support the other BATTLE specific aims with tumor response data, tissue specimens, and biomarker information.

Aim 2.1: We conclude that amphiregulin (AR) is the agonist in tumor cell lines (both lung cancer and head and neck cancer cell lines) with wild type EGFR that is associated with response to gefitinib and cetuximab. These agonists and the determination of whether EGFR is mutated will need to be studied in the tumor specimens from the patients participating in the BATTLE phase II trial of erlotinib to see if these *in vitro* findings translate into the clinical specimens available from the patients participating in this erlotinib study.

Aim 2.2: Our findings indicate the need for integration of IGF-1R-targeted agents to treatment regimens using EGFR TKIs for patients with lung cancer.

Aim 2.3: This data generated during the first year of our study supports our hypothesis that blood-based assays may provide markers of activity as well as potential predictive markers for selecting patients. In the upcoming year, we will be applying these methods to the batched samples collected to date from the BATTLE protocol to further investigate these markers, particularly for the VEGF inhibitors sorafenib and ZD6474.

Aim 2.4: We conclude that the majority of NSCLC cases express nuclear RXRα but expression of the two other RXR receptors (RXRβ and RXRγ) is very limited. Also, the use of bexarotene as a single agent in NSCLC appears to be limited and combination therapy will be tested.

Aim 3: In the next year, we will perform whole genome expression analysis in the first 50 tumor tissues to identify potential signatures associate with biomarker status measured in the Pathology Core. Potential signatures with strong association with clinical parameters, particularly PFS, will be emphasized. We will also construct the first panel of reverse-phase protein microarrays from the tissues and the corresponding serum. The first priority of the tissue use will be given to gene expression microarrays, followed by reverse-phase protein microarrays. Remaining tissues will be used for protein marker discovery using antibody arrays and 2-DE strategies as outlined in our research proposal. Selected candidate genes/proteins will be validated using quantitative RT-PCR and reverse-phase protein microarrays.

Aim 4: We conclude that the combination of rapamycin and Tarceva synergistically inhibited the growth of human NSCLC cells, thus supporting our hypothesis that co-targeting PI3K/Akt activation and suppression of mTOR signaling will result in enhanced anticancer activity; our study also reveals the expression of ASK1 in lung cancer cells and the action of Hsp90 inhibitor in blocking Akt phosphorylation. These data support our proposal to examine the role of ASK1 signaling pathway in mediating rapamycin-induced therapeutic effect.

Biostatistics and Data Management Core: In collaboration with clinical investigators, research nurses, the Biomarker Core, and basic scientists, the Biostatistics and Data Management Core has delivered the biostatistics and data management support as proposed.

Biomarker Core: The Biomarker Core has successfully combined standard methods of histopathology processing and assessment of lung cancer tissue samples with more advanced tools of molecular and genetic biomarker analyses for prospectively examining molecular biomarkers for individualized targeted therapy in NSCLC patients.

REFERENCES

Cappuzzo F, Hirsch FR, Rossi E et al., Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small cell lung cancer. *J Natl Cancer Inst* 97:643-55, 2004.

Cappuzzo F, Magrini E, Ceresoli GL, Bartolini S, Rossi E, Ludovini V, Gregorc V, Ligorio C, Cancellieri A, Damiani S, Spreafico A, Paties CT, Lombardo L, Calandri C, Bellezza G, Tonato M, Crino L. Akt phosphorylation and gefitinib efficacy in patients with advanced non-small-cell lung cancer. *J Natl Cancer Inst* 96:1133-41, 2004.

Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* 22:27-55, 1984.

DiGiovanni J, Bol DK, Wilker E, Carbajal S, Ramirez A, Jorcano J, Kiguchi K. Constitutive expression of insulin-like growth factor-1 in epidermal basal cells of transgenic mice leads to spontaneous tumor formation. *Cancer Res* 60(6):1561-70, 2000.

Duda DG, Cohen KS et al. "Differential CD146 expression on circulating versus tissue endothelial cells in rectal cancer patients: implications for circulating endothelial and progenitor cells as biomarkers for antiangiogenic therapy." *J Clin Oncol* 24(9):1449-53, 2006.

Giaccone G, Johnson DH, Manegold C et al. A phase III clinical trial of ZD1839 ("Iressa") in combination with gemcitabine and cisplatin in chemotherapy naive patients with advanced non-small-cell lung cancer (INTACT 1). *Ann Oncol* 13(Suppl 5):2-3, 2002.

Herbst RS, Giaccone G, Schiller JH et al. Gefitinib in combination with paclitaxel and carboplatin in advanced non-small-cell lung cancer: a phase III trial–INTACT 2. *J Clin Oncol* 22: 785-94, 2004.

Herbst RS, Prager D, Hermann R, Miller V, Fehrenbacher L, Hoffman P, Johnson B, Sandler AB, Mass R, Johnson DH. TRIBUTE – A phase III trial of erlotinib HCl (OSI-774) combined with carboplatin and paclitaxel (CP) chemotherapy in advanced non-small cell lung cancer (NSCLC). *Proc Am Soc Clin Oncol* 22: 619. 2004.

Herbst RS, Sandler AB. Overview of the current status of human epidermal growth factor receptor inhibitors in lung cancer. *Clin Lung Cancer* 6 Suppl 1:S7-S19, 2004.

Jemal A, Murray T, Samuels A, Ghafoor A, Ward E, Thun MJ. Cancer statistics, 2003. *CA Cancer J Clin* 53:5-26, 2003.

Jin DK, Shido K et al. "Cytokine-mediated deployment of SDF-1 induces revascularization through recruitment of CXCR4+ hemangiocytes." *Nat Med* 12(5):557-67, 2006.

Johnson D. ZD1839 ("Iressa") in combination with paclitaxel and carboplatin in chemotherapy-naive patients with advanced non-small cell lung cancer (NSCLC): results from a phase III clinical trial (INTACT II). *Ann Oncol* 13(Suppl 5):127, 2002

Kobayashi S, Boggon TJ, Dayaram T et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med* 352: 786-92, 2005.

Kulik G, Klippel A, Weber MJ. Antiapoptotic signaling by the insulin-like growth factor I receptor, phosphatidyl inositol 3-kinase, and Akt. *Mol Cell Biol* 17:1595-606, 1997.

Lin J, Adam RM, Santiestevan E, Freeman MR. The phosphatidylinositol 3'-kinase pathway is a dominant growth factor-activated cell survival pathway in LNCaP human prostate carcinoma cells. *Cancer Res* 59:2891-7, 1999.

Lynch TJ, Bell DW, Sordella R et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 350: 2129-39, 2004.

Mancuso P, Colleoni M et al. "Circulating endothelial-cell kinetics and viability predict survival in breast cancer patients receiving metronomic chemotherapy." *Blood* 108(2):452-9, 2006.

Miller VA, Kris MG, Shah N et al. Bronchioloalveolar pathologic subtype and smoking history predict sensitivity to gefitinib in advanced non-small-cell lung cancer. *J Clin Oncol* 22: 1103-09, 2004.

Norden-Zfoni A, Desai J et al. "Blood-based biomarkers of SU11248 activity and clinical outcome in patients with metastatic imatinib-resistant gastrointestinal stromal tumor (GIST)." *Clin Can Res* 13(9), in press, 2007.

Paez JG, Jänne PA, Lee JC et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 304:1497-500, 2004.

Pao W, Miller V, Zakowski M et al. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci U S A* 101:13306-11, 2004.

Porras A, Álvarez AM, Valladares A, Benito M. p42/p44 Mitogen-activated protein kinases activation is required for the insulin-like growth factor-I/insulin induced proliferation, but inhibits differentiation, in rat fetal brown adipocytes. *Mol Endocrinol* 12:825-34, 1998.

Shepherd F, Pereira J, Ciuleanu TE et al. A randomized placebo-controlled trial of Erlotinib in patients with advanced non-small cell lung cancer (NSCLC) following failure of 1st line or 2nd line chemotherapy. A National Cancer Institute of Canada Clinical Trials Group (NCIC CTG) trial. *Proc Am Soc Clin Oncol* 22: 7022, 2004.

Shigematsu H, Lin L, Takahashi T et al. Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. *J Natl Cancer Inst* 97:339-46, 2005.

Toker A, Newton AC. Cellular signaling: Pivoting around PDK-1. *Cell* 103:185-8, 2000.

APPENDIX A

Publications

Heterodimerization of Insulin-like Growth Factor Receptor/Epidermal Growth Factor Receptor and Induction of Survivin Expression Counteract the Antitumor Action of Erlotinib

Floriana Morgillo, Jong Kyu Woo, Edward S. Kim, Waun Ki Hong, and Ho-Young Lee

Department of Thoracic/Head and Neck Medical Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas

Abstract

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) have been used to treat non–small cell lung cancer (NSCLC). However, the overall response rate to EGFR TKIs is limited, and the mechanisms mediating resistance to the drugs are poorly understood. Here, we report that insulin-like growth factor-I receptor (IGF-IR) activation interferes with the antitumor activity of erlotinib, an EGFR TKI. Treatment with erlotinib increased the levels of EGFR/IGF-IR heterodimer localized on cell membrane, activated IGF-IR and its downstream signaling mediators, and stimulated mammalian target of rapamycin (mTOR)–mediated *de novo* protein synthesis of EGFR and survivin in NSCLC cells. Inhibition of IGF-IR activation, suppression of mTOR-mediated protein synthesis, or knockdown of survivin expression abolished resistance to erlotinib and induced apoptosis in NSCLC cells *in vitro* and *in vivo*. Our data suggest that enhanced synthesis of survivin protein mediated by the IGFR/EGFR heterodimer counteracts the antitumor action of erlotinib, indicating the needs of integration of IGF-IR–targeted agents to the treatment regimens with EGFR TKI for patients with lung cancer. (Cancer Res 2006; 66(20): 10100-11)

Introduction

The 5-year survival rate for lung cancer patients remains extremely poor (≤15; ref. 1), underscoring the need for more effective treatment strategies. Recently, new therapeutic approaches targeting signaling pathways involved in cell proliferation, apoptosis, angiogenesis, and metastasis have been investigated (2). Among the many potential target pathways, the epidermal growth factor (EGF) receptor (EGFR) signaling pathway has been studied most extensively because EGFR overexpression has been observed in a number of solid tumors, including 40% to 80% of non–small cell lung cancers (NSCLC; ref. 3). The EGFR signaling pathway activates the phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) pathways, which play major roles in cell proliferation, survival, and transformation and in therapeutic resistance (4, 5). In addition, the EGFR pathway is implicated in angiogenesis, and cell invasion by its regulation of the expression and activity of matrix metalloproteinases (6, 7).

These findings indicate the therapeutic potential of inhibitors of EGFR tyrosine kinase activation. EGFR tyrosine kinase activity can be inhibited by antibodies against the extracellular domain of EGFR, such as cetuximab, or by small molecules that block the ATP binding site of the cytoplasmic domain, such as gefitinib (ZD1839, Iressa; AstraZeneca Pharmaceuticals, Macclesfield, United Kingdom) and erlotinib (Tarceva®; OSI Pharmaceuticals; Genentech, South San Francisco, CA). Both forms of EGFR inhibition have single-agent antitumor activity against previously treated NSCLC (3, 8–10). Erlotinib exhibits an antiproliferative effect at nanomolar concentrations and has induced apoptosis and reversible cell cycle arrest at G_1 (11). *In vivo* preclinical models have shown that erlotinib administration markedly reduces EGFR autophosphorylation and growth in human head and neck cancer xenografts (HN5 and A431 cells) in nude mice (11, 12). In addition, gefitinib, combined with standard chemotherapeutic agents and/or radiotherapy in preclinical studies, has inhibited EGFR activation, thus causing G_1 cell cycle arrest and contributing to synergistic growth inhibition (13).

Despite a similar chemical structure, these two EGFR tyrosine kinase inhibitors (TKIs) have provided contrasting results in phase III clinical trials, in which only erlotinib showed significantly improved survival compared with placebo (14–16). The response to gefitinib and erlotinib has been suggested to be associated with sex, smoking status, tumor histology, and somatic mutations of the EGFR ATP binding site (17, 18). Recent data have suggested that the insulin-like growth factor-1 receptor (IGF-IR) pathway is also implicated in the resistance of gefitinib and anti-EGFR monoclonal antibody (19, 20). However, to our knowledge, the mechanisms involved in the IGF-IR–mediated acquired resistance to erlotinib in NSCLC cells have not been completely defined. In this article, we report that erlotinib induce EGFR/IGF-IR heterodimerization on the cell membrane, transmitting a survival signal through IGF-IR and its downstream mediators PI3K/Akt and p44/42 MAPK to stimulate mammalian target of rapamycin (mTOR)–mediated synthesis of EGFR and antiapoptotic survivin proteins. Consequently, inactivation of IGF-IR, suppression of mTOR-mediated protein synthesis, or knockdown of survivin protein renders EGFR-overexpressing NSCLC cells sensitive to the erlotinib treatment.

Materials and Methods

Cells, reagents, and animals. The human NSCLC cell lines H596, H226B, H226Br, H460, H1299, A549, H358, H661, and H322 were from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD) in a humidified atmosphere with 5% CO_2. IGF and EGF were from R&D Systems (Minneapolis, MN). Erlotinib were prepared as 10 mmol/L stock solution in DMSO and stored at −20°C. LY294002

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

W.K. Hong is an American Cancer Society Clinical Research Professor. H-Y. Lee is a faculty member at the Graduate School of Biomedical Sciences.

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(an inhibitor of PI3K), PD98059 (an inhibitor of the MEK1), rapamycin, and AG1024, a TKI of IGF-IR, were from Calbiochem-Novabiochem (Alexandria, New South Wales, Australia); these inhibitors were prepared as 20 mmol/L stock solutions in DMSO and also stored at −20°C. Adenoviral vectors expressing survivin (Ad-survivin; ref. 21) or dnIGF-IR/482 [adenovirus-expressing, dominant-negative IGF-IR (Ad-dnIGF-IR)], a soluble extracellular domain of IGF-IR with an engineered stop codon at amino acid residue 482 (22), and control adenoviral vector [adenovirus-expressing empty vector (Ad-EV)] were amplified as described elsewhere (23). We confirmed increases in the levels of IGF-IR protein by Western blot assay with an antibody for the α-subunit (anti-IGF-IRα N-20, Santa Cruz Biotechnology, Santa Cruz, CA) using medium from the cells that were infected with Ad-dnIGF-IR because IGF-IR/482 has been shown to produce and release the truncated α-subunit of IGF-IR into the medium (22). The effect of the combination of erlotinib and Ad-dnIGF-IR on established s.c. tumor nodules was studied in athymic nude mice (Harlan Sprague-Dawley, Indianapolis, IN) in a defined pathogen-free environment. Six-week-old female mice were used in this study; mice with necrotic tumors or tumors ≥1.5 cm in diameter were euthanized.

Cell proliferation assay. Cells were treated with erlotinib, rapamycin, LY294002, PD98059, AG1024, Ad-dnIGF-IR, Ad-EV, or their combinations in the absence or presence of 10% FBS, EGF (50 ng/mL), or IGF (50 ng/mL). For the experiments with the viruses, cells were infected with 5 and 10 particle forming unit (pfu) for Ad-dnIGF-IR or Ad-EV, in serum-free medium for 2 hours and then incubated for 3 days in RPMI medium supplemented with 10% FBS in the absence or presence of the indicated concentrations of erlotinib. Cell proliferation was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The drug concentrations required to inhibit cell growth by 50% were determined by interpolation from the dose-response curves. For defining the effect of the combined drug treatments, any potentiation was estimated by multiplying the percentage of cells remaining by each individual agent. The synergistic index was calculated as previously described (24). In the following equations, A and B are the effects of each individual agent, and AB is the effect of the combination. Subadditivity was defined as %AB / (%A × %B) < 0.9; additivity was defined as %AB / (%A × %B) = 0.9-1.0; and supra-additivity was defined as %AB / (%A × %B) > 1.0.

Clonogenic growth assay. The anchorage-dependent clonogenic growth assay was done by seeding NSCLC cell lines into six-well plates at low density (≈3×10^3 cells per well). Cells were either left uninfected or infected with 5 or 10 pfu/cell of Ad-dnIGF-IR or Ad-EV, incubated for 72 hours with different concentrations of erlotinib (0.1, 1.0, and 5.0 μmol/L), AG1024 (5 μmol/L), or combinations of the two drugs in serum-free RPMI medium in the absence or presence of IGF (50 ng/mL). Cells were replated in six-well plates and cultured in growth medium for 7 to 10 days, in a humidified atmosphere with 5% CO_2, at 37°C, and then colonies were fixed with 0.1% Coomassie blue (Bio-Rad Laboratories, Hercules, CA) in 30% methanol and 10% acetic acid. We then counted the number of colonies with >50 cells. For the anchorage-independent clonogenic growth assay, ~3×10^3 cells were suspended in 0.75 mL of 0.22% soft agar that was layered on top of 1 mL of 1% solidified agar in each well of 24-well plates. The plates were then incubated for 10 to 15 days in serum-free RPMI medium containing 0.1 or 1.0 μmol/L concentrations of erlotinib in the absence or presence of 10% FBS or IGF (50 ng/mL). The medium was changed daily during this period, at the end of which tumor cell colonies measuring at least 80 μm were counted under using a dissection microscope.

Cell cycle and apoptosis assays. For cell cycle and apoptosis assays, both adherent and nonadherent cells were harvested, pooled, and fixed with 1% paraformaldehyde and 70% ethanol. For the cell cycle analysis, we stained cells with 50 μg/mL propidium iodide and determined the percentage of cells in specific cell cycle phases (G_1, S, and G_2-M) by using a flow cytometer equipped with a 488 nm argon laser (Epics Profile II; Beckman Coulter, Miami, FL). Approximately 1×10^4 cells were evaluated for each sample. Apoptosis was assessed with a flow cytometry–based terminal deoxyribonucleotide transferase–mediated nick-end labeling (TUNEL) assay processed with an APO-bromodeoxyuridine (APO-BrdUrd) staining kit (Phoenix Flow Systems, San Diego, CA); this assay was modified as previously described (25). Cells treated with DMSO were used as a negative control, and for a positive control, we used the HL-60 leukemic cells treated with camptothecin provided with the kit.

Establishment of resistant cell line. The H460 cell cultures were continuously exposed to erlotinib (10 μmol/L) in routine culture medium that was replaced every day for 5 months. Initially, H460 cell numbers were substantially reduced, and for the next 2 months, the surviving cells were passaged approximately every 10 days with a seeding ratio of 1:2. Cell proliferation slowly increased to allow a passage every 7 days with a seeding ratio of 1:4 over the next 2 months. A stable growth rate was reached after a total of 5 months with routine maintenance of the H460/TKI-R cells involving passage every 4 days with a seeding ratio of 1:8 of the confluent cell number.

Subcellular fractionation. The following procedures were done at 4°C. Cells were scraped into PBS [10 mmol/L sodium phosphate (pH 7.4) and 150 mmol/L NaCl] and then collected by centrifugation. Cell pellets were resuspended with 1 mL of hypotonic buffer [10 mmol/L Tris-HCl (pH 7.5), 1 mmol/L $MgCl_2$, 50 μg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L Na_3VO_4]; 10 minutes later, the cells were transferred to a Dounce homogenizer and further disrupted by 25 strokes with a tight-fitting pestle. The homogenate was adjusted to the indicated NaCl concentration from a 5 mol/L stock solution, and nuclei were removed by centrifugation at 1,700 × *g* for 5 minutes. The postnuclear supernatant was centrifuged again at 10,000 rpm for 20 minutes to remove the mitochondrial fraction; the postmitochondrial supernatant was centrifuged at 45,000 × *g* for 60 minutes. The supernatant fraction, representing the cytosolic fraction, was adjusted to 1% NP40 from a 10% stock solution. The pellet, representing the plasma membrane fraction, was gently rinsed with 1 mL PBS and then resuspended in 1 mL hypotonic buffer containing 1% NP40.

Immunoblotting and coimmunoprecipitation. NSCLC cells (1×10^6 cells/100 mm^3 dish) were either left uninfected or infected with Ad-EV (50 pfu/cell) or Ad-survivin (50 pfu/cell) and then left untreated or treated with various concentrations of erlotinib (0.1-10.0 μmol/L), AG1024 (5.0 μmol/L), LY294002 (10.0 μmol/L), PD98059 (10.0 μmol/L), rapamycin (1.0 μmol/L), or their combinations in growth medium that was changed daily. When growth factor stimulation was done, cells were cultured in serum-free medium for 1 day and then incubated in EGF (50 ng/mL) or IGF (50 ng/mL) for 15 minutes. For the small interfering RNA (siRNA) transfection, H460 cells in the logarithmic growth phase in six-well plates (5×10^5 cells per well) were transfected with 10 μL of 20 μmol/L survivin siRNA or control scrambled siRNA (Dharmacon Research, Lafayette, CO) using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA), according to the protocol of the manufacturer. After 24 hours of incubation in growth medium, erlotinib was added, and the cells were harvested after 3 days of incubation. Immunoprecipitations were done using 3 mg protein from the total cell lysates and 1 μg mouse monoclonal anti-EGFR antibody, mouse monoclonal anti-IGF-IR antibody (Oncogene Sciences, Uniondale, NY), or healthy preimmune serum anti-mouse for the negative control and by incubating overnight at 4°C. The immunocomplexes were precipitated with protein-G agarose (Pharmacia-LKB Biotechnology, Piscataway, NJ). The immunoprecipitates were resolved on 6% SDS-PAGE gels, followed by Western blotting as described elsewhere (25).

Metabolic labeling. Metabolic labeling was done with H460 and TKI-R cells (5×10^5 in six-well plates). Cells were washed in PBS and incubated in RPMI medium without methionine and cysteine (Sigma, St. Louis, MO) for 2 hours. Next, the medium was replaced with fresh medium containing methionine and cysteine, to final concentrations of 150 mg/L, and the cells were labeled with trans-^{35}S (0.5 mCi; ICN, MP Biomedicals, Irvine, CA). The cells were then treated with 0.1% DMSO or erlotinib (10 μmol/L) for 1, 3, 6, 12, and 24 hours. At harvesting time, the cells were washed in ice-cold PBS and lysed in radioimmunoprecipitation assay buffer. Lysates containing equal amounts of protein (100 μg) were immunoprecipitated using 1 μg of antibody to detect EGFR or 1 μg of antibody to detect survivin (both from Santa Cruz Biotechnology) and 30 μL of 50% slurry of protein G agarose beads (Pharmacia-LKB Biotechnology, Piscataway, NJ). The immunoprecipitates were washed five times with lysis buffer, separated by SDS-PAGE,

and analyzed autofluorographically. Cell extracts were also subjected to Western blot analysis for β-actin to ensure that equal amounts of protein had been used. Two independent experiments were done with similar results; representative results of one experiment are presented.

Northern blot analysis. H460 cells (1×10^6 in 10 mm^3 plates) were treated with erlotinib (10 μmol/L) for different times (0, 24, 48, and 72 hours). The total cellular RNA was isolated by the application of TRIzol. For the Northern blotting, 10 μg of the total cellular RNA prepared from each sample was subjected to electrophoresis on a 1% agarose gel containing 2% formaldehyde and then stained with ethidium bromide, photographed, transferred to a Z probe membrane (Bio-Rad Laboratories), and hybridized to an [α-^{32}P]dCTP-labeled EGFR cDNA probe.

***In vivo* model.** The effect of the combination of erlotinib and Ad-dnIGF-IR on established s.c. tumor nodules was studied in athymic nude mice (Harlan Sprague-Dawley) in a defined pathogen-free environment. Briefly, 6-week-old female nude mice were irradiated with 350 rad from a cesium-137 source and then were injected s.c. with 1×10^7 H1299 cells in 100 μL of growth medium at a single dorsal site. The mice were randomly assigned to one of four treatment groups, with each group containing eight mice. Group 1 (control mice) received 1× PBS and Ad-EV, group 2 received erlotinib and Ad-EV, group 3 received 1× PBS and Ad-dnIGF-IR, and group 4 received erlotinib and Ad-dnIGF-IR. Tumor growth was quantified by measuring the tumors in three dimensions with calipers for a total of 35 days. After the tumor volumes reached ~75 mm^3 (considered day 0), the mice were treated with p.o. administered erlotinib (100 mg/kg of body weight) twice a day. We chose this dosage of erlotinib because it had had no notable effect on H1299 tumor growth in preliminary experiments (data not shown). On day 23, when tumor volumes reached ~125 mm^3, each mouse was given a single intratumoral injection of 2×10^9 particles of Ad-dnIGF-IR or Ad-EV in 100 μL of PBS. Mice with necrotic tumors or tumors ≥1.5 cm in diameter were euthanized immediately. The results were expressed as the mean tumor volume ($n = 5$) with 95% confidence intervals (95% CI). On day 35, all mice were sacrificed and tumor tissues were collected from the xenografts to determine whether the combination of erlotinib and

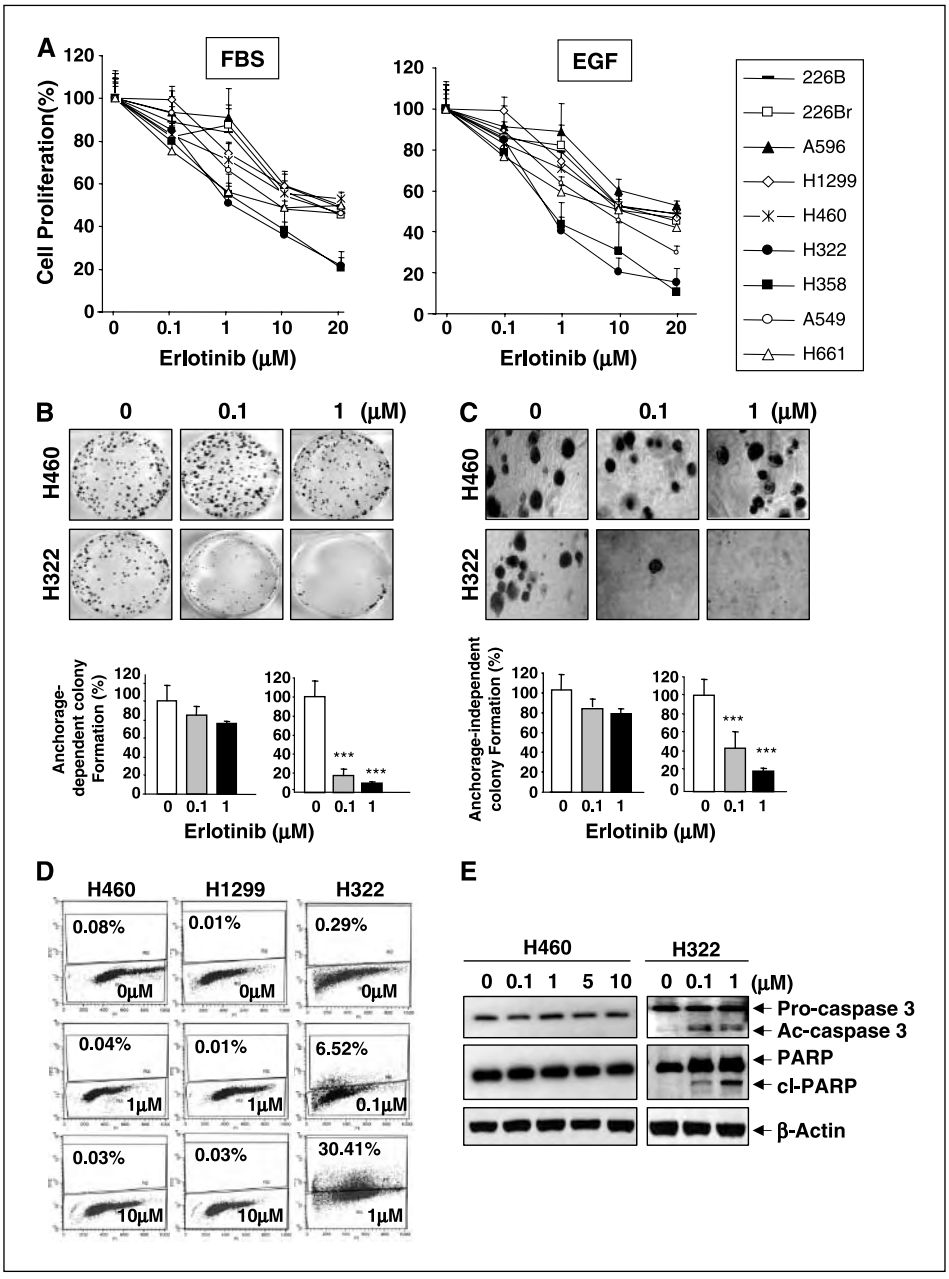

Figure 1. *A,* the MTT assay in NSCLC cell lines (H460, H1299, H661, A596, A549, H226B, H226Br, H322, and H358) treated with the indicated concentrations of erlotinib in RPMI 1640 containing 10% FBS or EGF for 3 days. *B,* clonogenic survival assay of H460 and H322 cells treated with the indicated concentrations of erlotinib. *C,* anchorage-independent growth assay of cells treated with the indicated concentrations of erlotinib. In (*A-C*), independent experiments were repeated thrice. *Columns,* mean of eight (*A*) or three (*B* and *C*) identical wells of a single representative experiment; *bars,* upper 95% CI; **, $P < 0.01$; ***, $P < 0.001$, for comparisons between erlotinib-treated and control cells. *D,* effects of the indicated concentrations of erlotinib on apoptosis in H322, H460, and H1299 NSCLC cells, assessed by a modified TUNEL assay. From a single representative experiment ($n = 2$). *E,* effects of the indicated concentrations of erlotinib on apoptosis-related enzyme expression in H322 and H460 NSCLC cells. The expression of caspase-3, PARP, and β-actin was assessed by Western blotting.

Ad-dnIGF-IR induced apoptosis *in vivo* by Western blot and immunohistochemical analyses as previously described (23).

Statistical analyses. The data acquired from the MTT assay were analyzed using Student's *t* test. All means and 95% CIs from eight samples were calculated using Microsoft Excel software (version 5.0; Microsoft Corporation, Seattle, WA). Cell survival comparisons among groups and statistical significance of differences in tumor growth in the combination treatment group and in the single-agent treatment groups were analyzed by ANOVA for 2×2 factorial design. All means from triplicate to eight samples and 95% CIs were calculated using SAS software (release 8.02; SAS Institute, Cary, NC). In all statistical analyses, two-sided *P* values of <0.05 were considered statistically significant.

Results

Differential apoptotic responses of NSCLC cells after treatment with erlotinib. To test the effects of EGFR TKIs on NSCLC cell proliferation, a subset of NSCLC cell lines (H460, H1299, H661, H596, H226B, H226Br, A549, H322, and H358) was treated with erlotinib in regular growth medium containing 10% FBS or in serum-free medium containing EGF. A MTT assay revealed that erlotinib has different levels of antiproliferative activities, depending on cell lines (Fig. 1*A*): Compared with the other NSCLC cell lines, H322 and H358 cells were more sensitive to the erlotinib treatment ($P < 0.001$). Approximately 1 μmol/L erlotinib significantly inhibited proliferation of H322 and H358 cells after 72 hours of treatment. In contrast, the drug concentrations required to inhibit cell growth by 50% for H460, H1299, H661, H596, H226B, H226Br, and A549 cells were 10 to 20 times higher than those needed to inhibit H322 and H358 cells. Consistent with the results from the MTT assay, erlotinib only slightly affected the anchorage-dependent and anchorage-independent colony-forming abilities of H460 and H1299 cells (data not shown) at concentrations <1 μmol/L, a concentration that significantly inhibited those abilities of H322 ($P < 0.001$; Fig. 1*B* and *C*; $P < 0.001$) and H358 (data not shown) cells.

We next asked whether the ability of 1 μmol/L erlotinib to inhibit H322 and H358 cell proliferation was due to decreased cell cycle progression and/or increased apoptosis. Flow cytometric analyses of propidium iodide–stained H460, H1299, and H322 cells revealed that treatment with 1 μmol/L erlotinib for 3 days resulted in no marked change in the cell cycle distribution (data not shown). However, fluorescence-activated cell sorter analysis followed by terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick-end labeling (TUNEL) staining revealed induction of apoptosis in 30.4% of the H322 cells treated with 1 μmol/L erlotinib. In contrast, treatment with up to 10 μmol/L erlotinib did not detectably increase the apoptotic population of H1299 and H460 cells (Fig. 1*D*). In agreement with these findings, the protein levels of the active form of caspase-3 (Ac-caspase-3) and the cleaved form of poly(ADP-ribose) polymerase (PARP; cl-PARP, the 89 kDa fragment) increased in the H322 cells treated with >0.1 μmol/L erlotinib but not in the H460 cells, even after treatment with 10 μmol/L erlotinib (Fig. 1*E*). H358 cells also responded with apoptosis to similar concentrations of erlotinib (data not shown). Together, these findings indicated the presence of mechanisms that affect the response of NSCLC cells to erlotinib-mediated apoptosis.

Role of IGF-IR signaling pathways in the development of resistance to erlotinib treatment in NSCLC cells. To investigate the mechanisms involved in the sensitivity of NSCLC cells to erlotinib, we first tested whether erlotinib successfully blocks activation of EGFR and its downstream mediators in NSCLC cell lines. Figure 2*A* shows that 0.1 to 1.0 μmol/L erlotinib suppressed the levels of phosphorylated EGFR (pEGFR), phosphorylated Akt (pAkt), and phosphorylated p44/42 MAPK (pp44/42 MAPK) in H460 and H1299 cells (cell lines that are weakly sensitive to erlotinib) and H322 and H358 cells (cell lines that are very sensitive to the drug). However, comparable induction of pAkt and p44/42 MAPK was evident in H460 and H1299 cells after treatment with >5.0 μmol/L erlotinib, doses that induce apoptosis in most of the H322 and H358 cells. pAkt and p44/42 MAPK are located in the nodal points of growth factor–mediated cell survival signaling, and the IGF-IR pathway can modulate the action of the erbB family blocking agents in various cancer cells (25–27). Hence, we tested whether IGF-IR was involved in increases in pAkt and p44/42 MAPK. Indeed, erlotinib concentrations >5.0 μmol/L induced phosphorylated IGF-IR (pIGF-IR) in H460 and H1299 cells but not in H322 and H358 cells.

We then studied the influence of IGF-IR signaling pathways on the response of NSCLC cells to erlotinib. H460 cells exhibited significantly decreased proliferation and anchorage-dependent and anchorage-independent colony-forming abilities when the cells were treated with erlotinib in serum-free medium compared with when they were treated in the presence of IGF-I. H322 cells, however, showed statistically significant sensitivity to erlotinib in all conditions (Fig. 2*B*), suggesting that the induced activation of the IGF-IR signaling pathway allows NSCLC cells to survive and proliferate when the EGFR pathway is blocked by erlotinib treatment.

To test our hypothesis, we compare the effects of erlotinib, either single or in combination with AG1024, an IGF-IR TKI, on the proliferation, clonogenic survival ability, and apoptosis of H460 cells. AG1024 has shown significantly lower affinity for the insulin receptor than for the IGF-IR (26). Combined treatment with erlotinib and AG1024 synergistically enhanced the antiproliferative effects of erlotinib on H460 cells compared with single treatment with each drug when cultured in complete (FBS) or serum-free medium in the absence or presence of IGF ($P < 0.001$; Fig. 2*C*; Supplementary Table S1). Erlotinib also showed significantly enhanced antiproliferative properties in H460 cells infected with an Ad-dnIGF-IR compared with the control cells infected with Ad-EV ($P < 0.001$; Fig. 2*D*). Moreover, combined treatment with erlotinib and AG1024 (Fig. 2*E*) or Ad-dnIGF-IR (Fig. 2*F*) significantly suppressed the anchorage-dependent, colony-forming ability of H460 cells ($P < 0.001$; Supplementary Table S2). Furthermore, TUNEL staining and flow cytometric analysis revealed that ~1% of control H460 cells, 1.2% of erlotinib-treated cells, and 26% (95% CI, 14.3-37.6%, $P < 0.05$) of AG1024-treated cells underwent apoptosis. In contrast, combined treatment with both erlotinib and AG1024 significantly enhanced TUNEL staining (77.1%; 95% CI, 64.4-89.8%; $P < 0.001$; Fig. 2*G*) and induced cleavage of the 113-kDa PARP to the 89-kDa fragment in parallel with the concomitant decreases in the levels of pIGF-IR, pAkt, and p44/42 MAPK (Fig. 2*H*). These findings suggest that the IGF-IR pathway provides an alternative proliferation and/or survival mechanism for NSCLC cancer cells in which EGFR is blocked by erlotinib.

Evidence of increased heterodimerization and membrane localization of IGF-IR and EGFR in erlotinib-treated H460 cells. We investigated the mechanism underlying NSCLC cell resistance to erlotinib using *in vitro* model of the H460 cell line (H460/TKI-R) that had been continuously treated with erlotinib. Treatment with >10 μmol/L erlotinib decreased the number of H460 cells, but proliferation of the remaining cells gradually

increased after 3 months of erlotinib treatment. Compared with the parent H460 cells, the H460 cells treated with 10 μmol/L erlotinib for 5 months (H460/TKI-R) had higher levels of pIGF-IR, pAkt, and pp44/42MAPK, with no detectable differences in the expression of IGF-IR, Akt, and p44/42MAPK (Fig. 3*A*). The dose-response curves in Fig. 3*B* revealed no detectable cytotoxicity from erlotinib treatment of the H460/TKI-R cells up to a concentration of 20 μmol/L, whereas inhibition of IGF-IR activation by the AG1024 treatment induced greater antiproliferative effects on the H460/TKI-R cells than it did on the H460 cells. Moreover, compared with a single agent, combined treatment with erlotinib and AG1024 significantly suppressed the anchorage-dependent, colony-forming ability of H460/TKI-R as well as H460 cells ($P < 0.001$; Fig. 3*C*). The effects of combined treatment with erlotinib and AG1024 on colony formation was greater in H460/TKI-R cells than in H460 cells, indicating the dependence of H460/TKI-R cells on the IGF-IR signaling pathway for maintaining cell proliferation and tumorigenic potential.

We investigated the mechanism of erlotinib-mediated activation of IGF-IR in H460 cells. One mechanism by which the growth factor receptor is activated in tumor cells is by receptor dimerization; another is by overwhelming negative regulatory mechanisms that suppress receptor activation. Recent studies have revealed the interaction between the ErbB receptor families and IGF-IR in several tumor models, including breast cancer and oral cancer cell lines (19, 28–32). We, therefore, tested whether EGFR interacts with IGF-IR in H460/TKI-R cells by performing immunoprecipitation. EGFR immunoprecipitates from H460/TKI-R cells showed greater IGF-IR binding compared with that from the parental H460 cells (Fig. 3*D*). Control immunoprecipitates using preimmune serum exhibited no immunoreactive band. The interaction between EGFR and IGF-IR was observed as early as 30 minutes after the erlotinib treatment (Fig. 3*E*). In contrast, no detectable change was observed in the levels of EGFR-EGFR or EGFR-ErbB2 interaction in H460 cells treated with erlotinib for 3 days (Fig. 3*F, top*). Similarly, IGF-IR immunoprecipitates from erlotinib-treated H460 cells showed greater levels of EGFR binding than untreated cells did (Fig. 3*F, bottom*), whereas no detectable binding was observed when the IGF-IR immunoprecipitates were immunoblotted to ErbB2 or ErbB3. Increased EGFR/IGF-IR heterodimerization was also observed in H1299 cells treated with 10 μmol/L erlotinib (Fig. 3*G*). In contrast, the EGFR and control (preimmune serum) immunoprecipitates from untreated or erlotinib-treated H322 cells exhibited no immunoreactive band. These results suggested that erlotinib induces physical contact between EGFR and IGF-IR, which is accumulative.

Erlotinib treatment–induced expression of survivin protein protects NSCLC cells from apoptosis. We next attempted to find evidence connecting erlotinib-induced activation of the IGF-IR with survival of NSCLC cells. Because the inhibitor of apoptosis

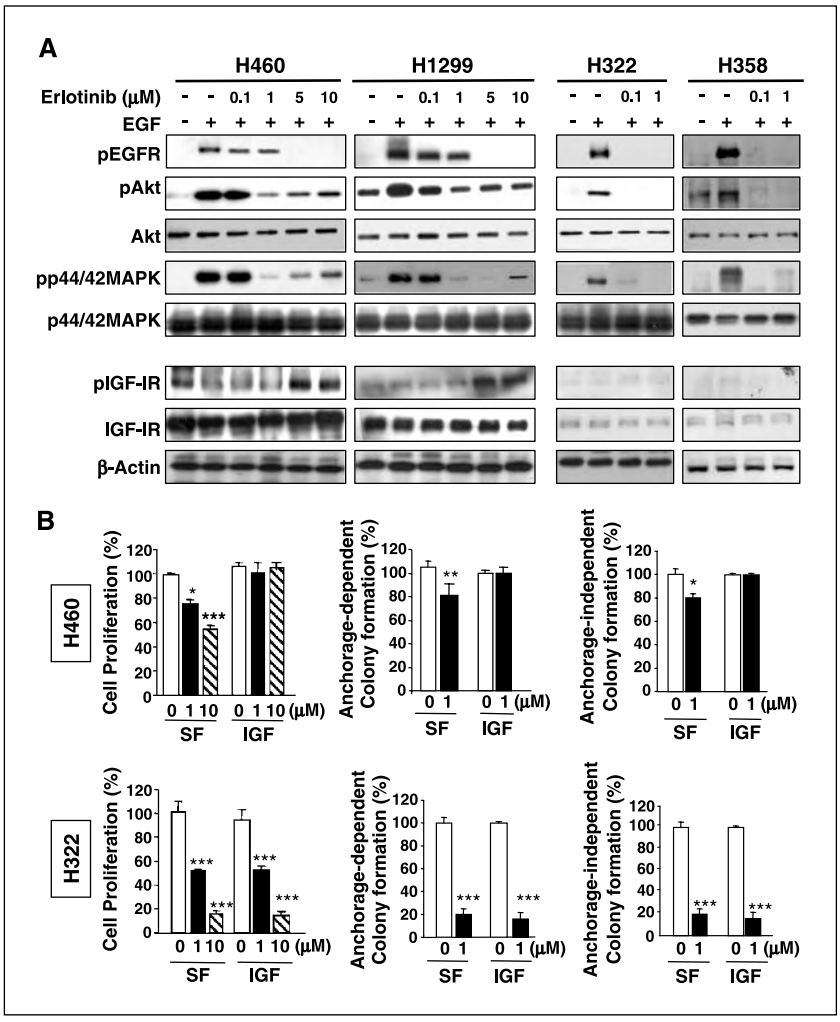

Figure 2. *A,* immunoblotting of the EGFR, IGF-IR, and their downstream signaling components in NSCLC cells treated with indicated concentrations of erlotinib. Western blotting on β-actin is included as a loading control. *B,* role of the IGF-I on the proliferation and survival of NSCLC cells. *Left,* MTT assay in H460 and H322 cells incubated in the serum-free medium without (*SF*) or with IGF-I (50 ng/mL) in the presence of indicated concentrations of erlotinib for 3 days. *Middle* and *right,* efficacy of the indicated concentrations of erlotinib in inhibiting anchorage-dependent and anchorage-independent growth of H460 and H322 cells, respectively, in serum-free and IGF-dependent conditions.

proteins, including survivin and XIAP, decrease the sensitivity of tumor cells to chemotherapeutic drugs, thereby conferring resistance to apoptosis (21, 33), we tested the effects of erlotinib on the expression of these proteins in a subset of NSCLC cells with weak or great sensitivity to erlotinib. We found that expression of survivin but not XIAP markedly increased in H1299, H460, H661, A549, A596, and 226B cells (Fig. 4*A, top*) during the time IGF-IR was phosphorylated by the erlotinib treatment (Fig. 4*A, bottom*). In contrast, H358 and H322 cells showed no detectable changes in the protein levels of survivin, XIAP, and pIGF-IR during the time EGFR was inactivated by erlotinib treatment. Erlotinib induced survivin expression in a time- and dose-dependent manner (Fig. 4*B*). Interestingly, a similar but less pronounced increase in EGFR expression was observed in H1299, H460, H661, A549, H596 H226Br, and H226B, H460/TKI-R cells but not in H358 and H322 cells. Increases in the survivin and EGFR expression were also observed in H460/TKI-R cells (Fig. 4*C*).

We then tested the response of H460 cells, in which survivin expression was abolished by siRNA transfection. Western blot analysis revealed an obvious increase in PARP cleavage in the 460 cells by the treatment with erlotinib (Fig. 4*D*). Among H322 cells, in which survivin overexpression was induced by the infection with Ad-survivin, the PARP cleavage was substantially reduced after the erlotinib treatment (Fig. 4*E*). These results indicated that increased expression of survivin protein protected NSCLC cells from the erlotinib-induced apoptosis.

mTOR pathway induces *de novo* protein synthesis of EGFR and survivin and protects NSCLC cells from apoptosis. We investigated the mechanisms of erlotinib-mediated increase in survivin and EGFR protein expression. According to Northern blot analysis, exposure of H460 cells to erlotinib resulted in no change in the mRNA levels of survivin (Fig. 5*A*) and EGFR (data not shown). We then determined the effects of erlotinib on the rates of survivin and EGFR protein synthesis. Metabolic labeling of the H460 cells with [^{35}S]Met-Cys revealed that the rate of [^{35}S]labeled survivin (Fig. 5*B*) and EGFR (data not shown) synthesis was remarkably greater in the erlotinib-treated H460 and H460/TKI-R cells than in the untreated parental H460 cells. We then determined whether

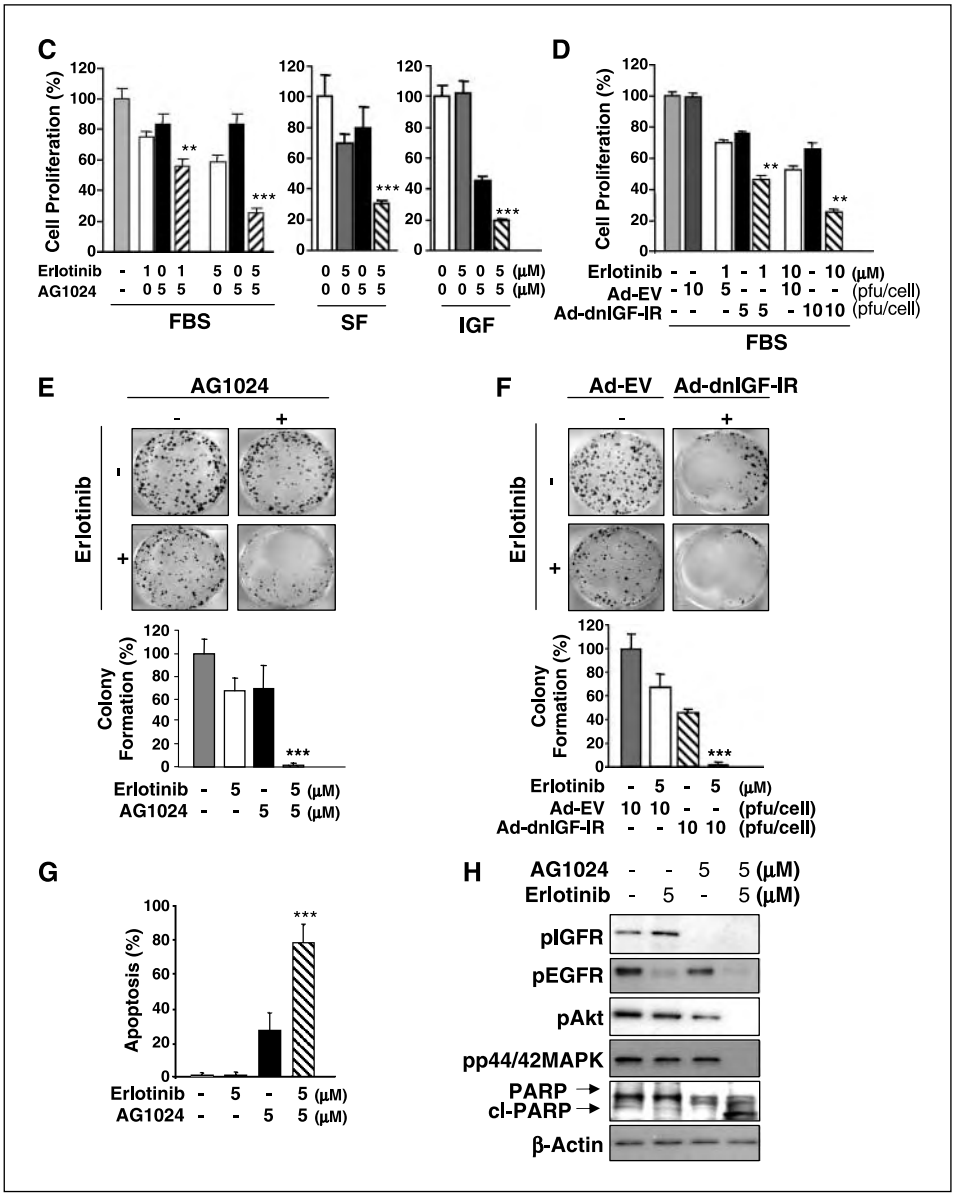

Figure 2 *Continued.* *C* and *D,* effect of targeting both the EGFR and the IGFR-IR on cell proliferation. MTT assay in H460 cells uninfected (*C*) or infected with 5 or 10 infectious forming unit of Ad-EV or Ad-dnIGF-IR (*D*) and treated with indicated concentrations of erlotinib, AG1024, or their combination in serum-free RPMI 1640 containing 10% FBS or IGF (50 ng/mL) for 3 days. *E* and *F,* survival of H460 cells treated with erlotinib (5 μmol/L), AG1024 (5 μmol/L), or their combination (*E*), or cells infected with 10 pfu/cell of Ad-EV or Ad-dnIGF-IR and then untreated or treated with erlotinib (5 μmol/L; *F*) were assessed by counting colonies consisting of >50 cells after 10 days of growth. *G* and *H,* effects of 5 μmol/L erlotinib, 5 μmol/L AG1024, or their combination on apoptosis (*G*) and expression of pEGFR, pIGF-IR, pp44/42MAPK, and pAkt (*H*) were analyzed in H460 cells by a flow cytometry–based TUNEL assay and Western blotting. β-Actin, loading control. *Columns,* mean value of eight (MTT) or three (clonogenic growth assay and TUNEL assay) identical wells of a single representative experiment ($n = 3$); *bars,* upper 95% CI (*B-G*). ***, $P < 0.001$ for comparisons between cells treated with drug combination and cells treated with single agent.

mTOR is involved in the erlotinib-induced protein synthesis of survivin and EGFR by determining the levels of phosphorylated 4E-BP1 and $p70^{s6k}$, downstream mediators of mTOR (27, 34), in the H460 cells treated with erlotinib alone or in combination with rapamycin, an mTOR inhibitor, or AG1024 for 3 days. Western blot analysis revealed that erlotinib up-regulated the protein levels of p4E-BP1, $pp70^{s6k}$ in association with increases in survivin and EGFR expression, all of which were suppressed by

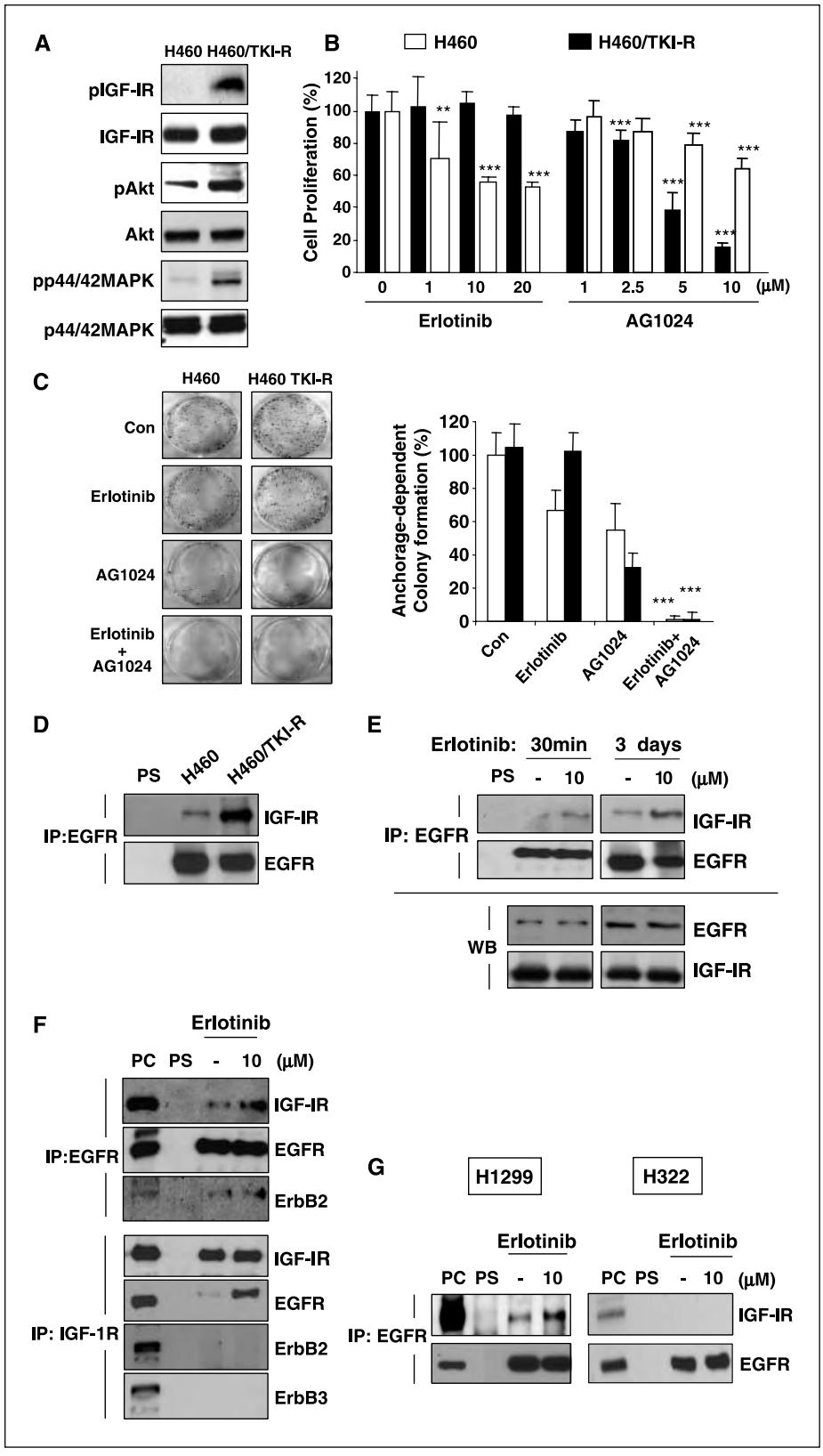

Figure 3. *A,* Western blot analysis of indicated protein expressions in H460 and H460/TKI-resistant (H460/TKI-R) cells. *B,* MTT assay on the proliferation of H460 and H460/TKI-R cells treated with erlotinib (1-20 μmol/L) or AG1024 (1-10 μmol/L). Cells treated with 0.1% DMSO were included as a control (0). *C,* survival of H460 and H460/TKI-R cells treated with erlotinib (5 μmol/L), AG1024 (5 μmol/L), or their combination. *Columns,* mean value of eight (MTT) or three (clonogenic assay) identical wells of a single representative experiment ($n = 3$); *bars,* upper 95% CI (*A* and *B*). ***, $P < 0.001$ compared with control. *D* to *G,* coimmunoprecipitation was done for the interaction between EGFR and IGF-IR. Whole-cell extracts from H460 and H460/TKI-R cells (*D*) or H460 cells, H1299, and H322 cells untreated or treated with erlotinib (10 μmol/L) for 30 minutes (*E*) or 3 days (*E-G*) were immunoprecipitated (*IP*) with anti-EGFR or anti-IGF-IR antibodies. The immunoprecipitates were subjected to Western blot analysis with indicated antibodies. Input (*PC*) represents cell lysates that were not subjected to immunoprecipitation. Control immunoprecipitation was done using control mouse preimmune serum (*PS*).

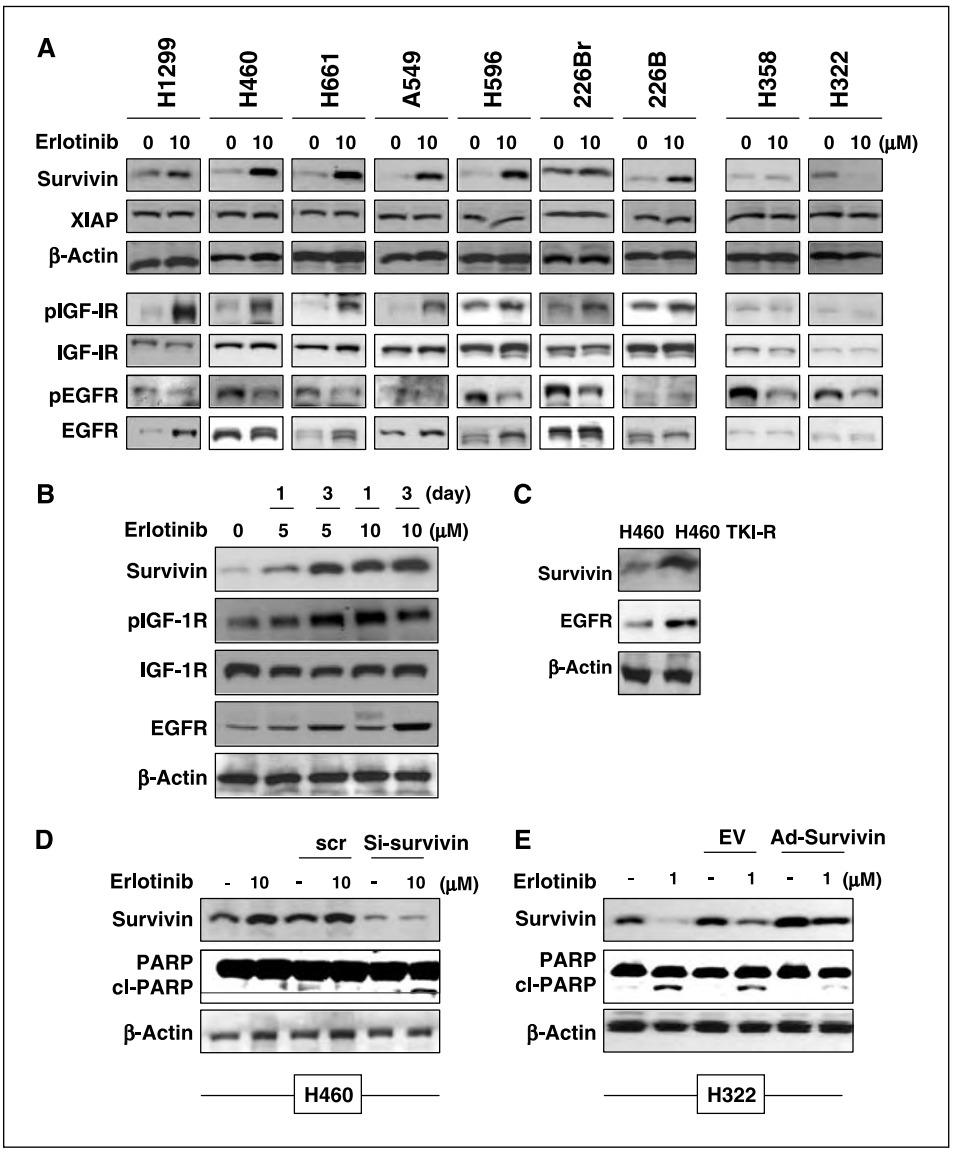

Figure 4. *A* and *B,* Western blot analysis on the survivin, XIAP, pIGF-IR, IGF-IR, pEGFR, EGFR protein expression in indicated NSCLC cell lines treated with erlotinib (1-10 μmol/L) for 1 (*B*) or 3 (*A, B*) days. *C,* Western blot analysis on the survivin and EGFR protein in H460 TKI/R cells. *D,* effect of the knockdown of survivin expression on H460 cells in the presence of erlotinib. H460 cells transfected with scramble (*scr*) or survivin siRNA (*si-survivin*), untreated and treated for 48 hours with erlotinib (10 μmol/L), were subjected to protein extraction and Western blotting for evaluation of caspase-3 (pro-caspase-3) and PARP. Loading control: β-actin. *E,* effects of overexpression of survivin in erlotinib-induced apoptosis in H322 cells. H322 cells were infected with 50 pfu/cell of control virus (Ad-EV) or Ad-survivin and incubated for 3 days in the presence of erlotinib (1 μmol/L). Protein extract was subjected to Western blotting for evaluation of survivin, caspase-3 (pro-caspase-3), and PARP. Loading control: β-actin.

treatment with AG1024 or rapamycin (Fig. 5*C*). Moreover, the combined treatment with AG1024, LY294002 (PI3K inhibitor), PD98059 (MEK inhibitor), or rapamycin reduced the levels of membranous EGFR expression (Fig. 5*D*) and of the EGFR and IGF-IR heterodimer (Fig. 5*E*) induced by the 3 days treatment of erlotinib. These findings suggested that the increases in the levels of EGFR/IGF-IR heterodimer on cell membrane and protein expressions of survivin and EGFR were mediated at least in part through translation-dependent events mediated by IGF-IR signaling pathways. We then tested whether inhibitors of the IGF-IR and mTOR pathways sensitize the H460 cells to the erlotinib treatment. H460 cells treated with erlotinib and AG1024 (Fig. 2*G* and *H*) or rapamycin (Fig. 5*F*) showed an increase in the PARP cleavage, suggesting that suppression of IGF-IR and mTOR pathways could restore the apoptotic activities of erlotinib in NSCLC cells.

Antitumor efficacy of dual targeting of EGFR and IGF-IR signaling pathways *in vivo*. To determine whether the inhibition of IGF-IR signaling can enhance the antitumor activities of erlotinib *in vivo*, we tested the effects of erlotinib, Ad-dnIGF-IR, and their combination on the growth of H1299 NSCLC xenograft tumors established in athymic nude mice. The mice treated with erlotinib plus Ad-dnIGF-IR showed synergistically reduced tumor growth compared with the control mice or the mice treated with erlotinib or Ad-dnIGF-IR alone (Fig. 6*A*; Supplementary Table S3). At the end of the study, the mean tumor volume in combined treatment group was 23% ($P < 0.001$) of the mean volume in the control group. Thus, the combination of erlotinib and Ad-dnIGF-IR enhanced the antitumor effects on the growth of NSCLC cells *in vivo*.

We then determined the effects of erlotinib, Ad-dnIGF-IR, and their combination on the activation of the IGF-IR and EGFR, the expression of survivin and EGFR, and the induction of apoptosis *in vivo*. According to Western blot analysis of total protein extracts harvested from the H1299 xenograft tumor tissues, the levels of pEGFR were decreased by erlotinib. In addition, erlotinib treatment induced marked increases in the levels of pIGF-IR, EGFR, and survivin, all of which were effectively blocked by Ad-dnIGF-IR (Fig. 6*B*). Combined treatment with erlotinib and Ad-dnIGF-IR also increased the levels of Ac-caspase-3, which is confirmed by the

immunohistochemical staining of the H1299 xenograft tumor tissues (Fig. 6*C*). Together, these findings suggested that the combined treatment with erlotinib and Ad-dnIGF-IR exert enhanced *in vivo* antitumor activities by decreased expression of survivin and EGFR and induction of apoptosis.

Discussion

Several preclinical and clinical discoveries have associated EGFR TKIs with antitumor activities. However, the limited response rates of patients to EGFR TKIs, even in patients with high levels of EGFR (15, 16, 35), have been raising questions about the mechanisms leading to the EGFR TKI resistance. Although somatic mutations of the EGFR ATP binding site have been associated with the response to the EGFR TKIs in some cases (17, 18), increasing number of evidence have suggested that the presence of other pathways that mediate the resistance of cancer cells to EGFR TKI therapy (36, 37). In this article, we have shown, to our knowledge for the first time, that erlotinib induces survival of NSCLC cells by inducing heterodimerization of EGFR/IGF-IR, activating IGF-IR pathway and its downstream mediators Akt and p44/42 MAPK, and thus stimulating mTOR-mediated protein synthesis of survivin that plays a crucial role in the blocking apoptosis. We showed here that the blockade of the IGF-IR to mTOR signaling pathway was sufficient to suppress *de novo* survivin protein synthesis and to restore apoptotic activities of erlotinib in NSCLC cells *in vitro* and *in vivo*. Our data present clear evidence that crosstalk between the IGF-IR and the EGFR signaling pathways and consequential survivin expression are involved in the NSCLC cell resistance to erlotinib. The erbB2/Her2/neu, a known preferred coreceptor for the EGFR, has been suggested to play a role in inducing NSCLC cell survival against EGFR TKIs (28). However, we found that the erbB2/Her2/neu was inactivated by the EGFR TKIs in NSCLC cells (data not shown), consistent with previous reports (38). In addition, an interaction between IGF-IR and erbB2 was undetectable, regardless of erlotinib treatment. Therefore, erbB2/Her2/neu is not likely to have a role in inducing EGFR TKI resistance in NSCLC cells.

We investigated the detailed mechanism that mediates IGF-IR activation by erlotinib and the consequent development of drug resistance. Given that gefitinib-resistant DU145/TKI-R prostate cells have shown considerably higher basal levels of IGF-II mRNA than wild-type cells (19), erlotinib might have increased the expression of IGF and conferred resistance to the drug to NSCLC cells. However, in gefitinib-resistant breast cancer cell lines, the IGF II mRNA level did not differ from that of the original clone (19), indicating that autocrine/paracrine production is not entirely responsible for the sensitivity of the cell to EGFR TKIs. Perhaps our most striking finding was that in H460 and H1299 cells, erlotinib induced heterodimerization between IGF-IR and EGFR. The interaction between EGFR and IGF-IR also has been observed in cancer cells (29–31). Given the considerable similarity between EGFR and IGF-IR in the sequence of their extracellular domain (39) and their reliance on EGF and IGF to achieve cell cycle progression and survival, it is plausible that erlotinib-mediated IGF-IR/EGFR heterodimerization can stimulate intracellular signaling components in a distinct pattern and allow NSCLC cells to resist the drug.

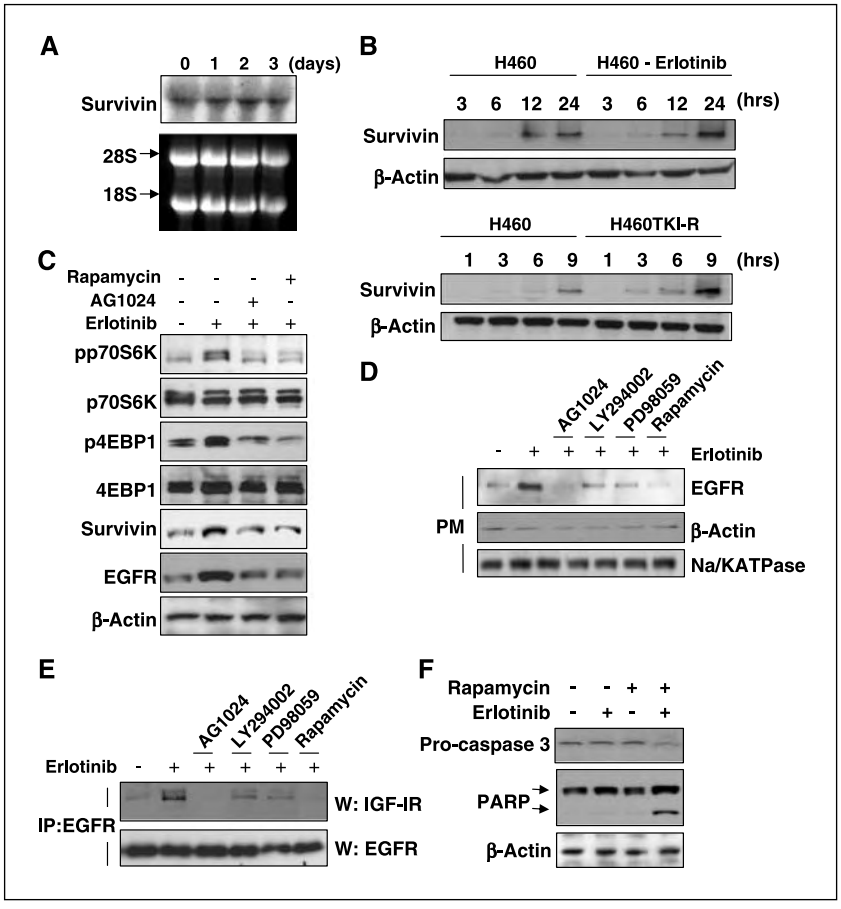

Figure 5. *A,* Northern blot analysis on survivin mRNA in H460 treated with erlotinib (10 μmol/L) for 1, 2, or 3 days. *B,* survivin and EGFR protein synthesis evaluated by metabolic labeling in untreated 460 cells, H460 cells treated with erlotinib (10 μmol/L), and H460TKI-R cells. Cell extracts were also subjected to Western blot analysis for β-actin to ensure that equal amounts of protein were used. *C,* expression of phosphorylated p70S6K (pp70S6k), p70S6K, phosphorylated 4EBP1 (p4EBP1), 4EBP1, survivin and EGFR in H460 cells treated with erlotinib (10 μmol/L), either single or in combination with AG1024 (5 μmol/L) or rapamycin (1 μmol/L), for 3 days. *D* and *E,* effects of erlotinib in combination with AG1024, LY294002, PD98059, or rapamycin on the plasma membrane (*PM*) localization of EGFR (*D*) and on the interaction between EGFR and IGF-IR (*E*). β-Actin, control for cytosol fraction; Na/K ATPase, control for plasma membrane fraction. *F,* effect of combined treatment with erlotinib (10 μmol/L) and rapamycin (1 μmol/L) for 3 days on apoptosis in H460 cells. Protein extract was subjected to Western blotting for the evaluation of pro-caspase-3 and PARP. Loading control: β-actin.

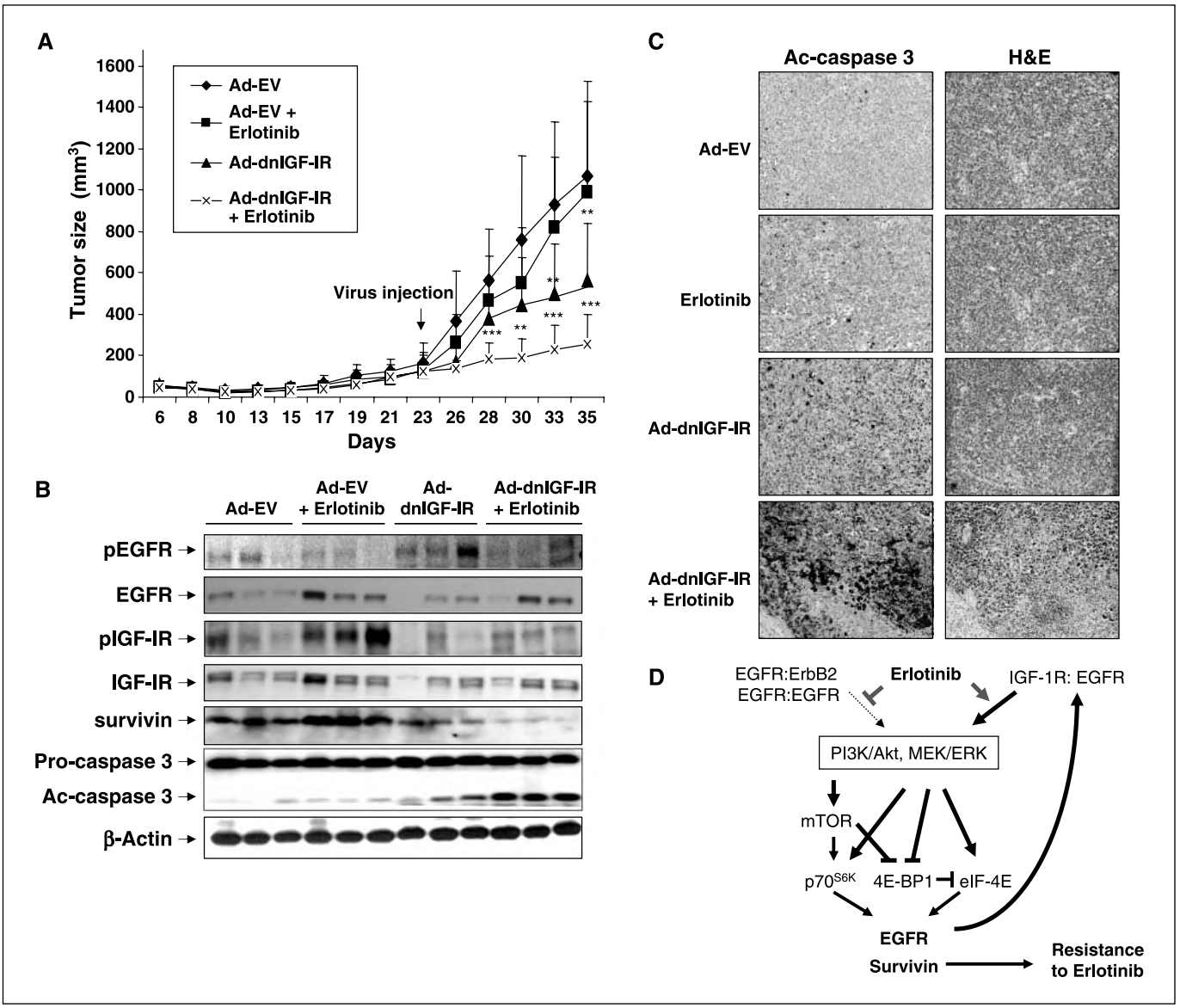

Figure 6. Effects of combined treatment with erlotinib and recombinant Ad-dnIGF-IR on growth of H1299 NSCLC xenograft tumors in athymic nude mice. The mice were randomly assigned to one of four treatment groups, with each group containing five mice. Group 1 (control mice) received 1× PBS and Ad-EV, group 2 received erlotinib and Ad-EV, group 3 received 1× PBS and Ad-dnIGF-IR, and group 4 received erlotinib and Ad-dnIGF-IR. *A,* effect of erlotinib (40 mg/kg body weight, administered p.o. twice daily) on tumor volume. When tumor volume was ~125 mm^3, mice were treated with Ad-IGF-IR or Ad-EV (control) in 100 μL PBS. *Points,* mean tumor volume (n = 5) with 95% CI; *bars,* SE. **, $P < 0.01$, ***, $P < 0.001$ for comparisons between drug-treated and control cells for each series of experiments. *B,* effects of erlotinib and Ad-dnIGF-IR on the expression of pEGFR, EGFR, pIGF-IR, IGF-IR, survivin, and pro-caspase-3 and Ac-caspase-3 in NSCLC xenograft tumors, assessed by Western blotting. β-Actin = loading control. *C,* effects of combined erlotinib and Ad-IGF-IR on expression of Ac-caspase-3. Tissues were stained with H&E. Representative section from each condition. *D,* schematic model of resistance mechanism to erlotinib.

Previous studies have shown the ability of erlotinib to induce EGFR mRNA and protein expression in the erlotinib-resistant biliary tract cancer cell line HuCCT1 but not in the susceptible A431 epidermoid cell line (40). In current study, we found that erlotinib induces mTOR-mediated *de novo* protein synthesis of survivin and EGFR with no detectable change in their mRNA levels, indicating diverse responses of different cancer cells to erlotinib. Our results may explain some apparently paradoxical findings in several clinical trials (41), in which up-regulation of pEGFR was observed after treatment of breast cancer patients with erlotinib. In another report, modifications of EGFR serum values during treatment of NSCLC seemed to reflect gefitinib activity; responding patients showed decreased serum levels of EGFR relative to those in patients with refractory disease (42). Our data also show the critical role of the induced survivin proteins in the development of resistance to erlotinib; (*a*) survivin expression was induced in NSCLC cell lines with weak sensitivity to the erlotinib treatment; (*b*) overexpression of survivin protected the sensitive NSCLC cells from erlotinib-induced apoptosis; and (*c*) knockdown survivin expression by siRNA provoked apoptosis in NSCLC cells with weak erlotinib sensitivity. mTOR has been known to regulate the translation of subsets of mRNA, many of which encode for proteins involved with driving cell growth, proliferation, and angiogenesis (43). Therefore, resistance and sensitivity to erlotinib in NSCLC may be determined at least in part by the ability of the cancer cells to stimulate mTOR-mediated synthesis of specific proteins that

have key roles in cell proliferation and/or survival and thus to adapt to a stressful environment.

In summary, our findings provide definitive *in vitro* and *in vivo* evidence that erlotinib induces heterodimerization of the EGFR/IGF-IR and stimulates IGF-IR and downstream pathways, including PI3K/Akt, MEK/extracellular signal-regulated kinase (ERK), resulting in the mTOR-mediated increases in EGFR and survivin proteins. On the basis of these findings, it is plausible to suggest that increased EGFR proteins further enhance the interplay between the EGFR and IGFR on the cell membrane, resulting in a further amplification of IGF-IR to mTOR signaling. In addition, the increased survivin proteins seem to provide survival potential to the NSCLC cells against the erlotinib treatment (Fig. 6*D*).

Our findings have direct effect to the treatment of NSCLC with erlotinib. The data showing no detectable interaction between EGFR/IGF-IR in NSCLC cell lines with low levels of IGF-IR expression suggests that the expression of IGF-IR is an important factor for the EGFR and IGF-IR complex and erlotinib sensitivity. Therefore, IGF-IR expression may serve as a predictor for erlotinib resistance in NSCLC. IGF-IR signaling pathway also plays a key role in the resistance to several therapeutic drugs (12, 27, 44–47). Overexpression of IGF-IR has been observed in various human cancers, including lung cancer (48), and is associated with a poor prognosis (49). Importantly, our unpublished data showed that clinical samples from NSCLC patients revealed that the majority of EGFR-overexpressing samples showed correlative increases in IGF-IR protein levels compared with their paired normal counterparts from the same patients. With this prospect, IGF-IR–targeting combination treatment may be required when erlotinib is considered as a therapeutic agent for NSCLC patients. Alternatively, mTOR inhibitors could confer benefit to erlotinib-resistant patients. In light of this notion, we have shown that combined treatment with erlotinib and inhibitors of IGF-IR or mTOR suppressed survivin and EGFR expression, decreased proliferation of NSCLC cells, and induced apoptosis in NSCLC cells *in vitro* and *in vivo*. Further studies are warranted to validate whether the erlotinib combined with inhibitors of IGF-IR or mTOR inhibitors could enhance the objective response and survival rates in NSCLC patients.

Acknowledgments

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References

1. Jemal A, Murray T, Samuels A, Ghafoor A, Ward E, Thun MJ. Cancer statistics, 2003. CA Cancer J Clin 2003;53:5–26.

2. Ferreira CG, Huisman C, Giaccone G. Novel approaches to the treatment of non-small cell lung cancer. Crit Rev Oncol Hematol 2002;41:57–77.

3. Mendelsohn J. The epidermal growth factor receptor as a target for cancer therapy. Endocr Relat Cancer 2001;8:3–9.

4. Brognard J, Clark AS, Ni Y, Dennis PA. Akt/protein kinase B is constitutively active in non-small cell lung cancer cells and promotes cellular survival and resistance to chemotherapy and radiation. Cancer Res 2001;61:3986–97.

5. Brognard J, Dennis PA. Variable apoptotic response of NSCLC cells to inhibition of the MEK/ERK pathway by small molecules or dominant negative mutants. Cell Death Differ 2002;9:893–904.

6. Khazaie K, Schirrmacher V, Lichtner RB. EGF receptor in neoplasia and metastasis. Cancer Metastasis Rev 1993;12:255–74.

7. Ellerbroek SM, Halbleib JM, Benavidez M, et al. Phosphatidylinositol 3-kinase activity in epidermal growth factor-stimulated matrix metalloproteinase-9 production and cell surface association. Cancer Res 2001;61:1855–61.

8. Sandler A. Clinical experience with the HER1/EGFR tyrosine kinase inhibitor erlotinib. Oncology (Huntingt) 2003;17:17–22.

9. Ready N. Inhibition of the epidermal growth factor receptor in combined modality treatment for locally advanced non-small cell lung cancer. Semin Oncol 2005;32:S35–41.

10. Hidalgo M. Erlotinib: preclinical investigations. Oncology (Huntingt) 2003;17:11–6.

11. Moyer JD, Barbacci EG, Iwata KK, et al. Induction of apoptosis and cell cycle arrest by CP-358,774, an inhibitor of epidermal growth factor receptor tyrosine kinase. Cancer Res 1997;57:4838–48.

12. Pollack VA, Savage DM, Baker DA, et al. Inhibition of epidermal growth factor receptor-associated tyrosine phosphorylation in human carcinomas with CP-358,774: dynamics of receptor inhibition *in situ* and antitumor effects in athymic mice. J Pharmacol Exp Ther 1999;291:739–48.

13. Albanell J, Rojo F, Baselga J. Pharmacodynamic studies with the epidermal growth factor receptor tyrosine kinase inhibitor ZD1839. Semin Oncol 2001;28:56–66.

14. Shepherd FA, Rodrigues Pereira J, Ciuleanu T, et al. Erlotinib in previously treated non-small-cell lung cancer. N Engl J Med 2005;14:123–32.

15. Giaccone G, Herbst RS, Manegold C, et al. Gefitinib in combination with gemcitabine and cisplatin in advanced non-small-cell lung cancer: a phase III trial-INTACT 1. J Clin Oncol 2004;22:777–84.

16. Herbst RS, Giaccone G, Schiller JH, et al. Gefitinib in combination with paclitaxel and carboplatin in advanced non-small-cell lung cancer: a phase III trial-INTACT 2. J Clin Oncol 2004;22:785–94.

17. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004;350:2129–39. Epub 2004 Apr 29.

18. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304:1497–500. Epub 2004 Apr 29.

19. Jones HE, Goddard L, Gee JM, et al. Insulin-like growth factor-I receptor signalling and acquired resistance to gefitinib (ZD1839; Iressa) in human breast and prostate cancer cells. Endocr Relat Cancer 2004;11:793–814.

20. Liu B, Fang M, Lu Y, Mendelsohn J, Fan Z. Fibroblast growth factor and insulin-like growth factor differentially modulate the apoptosis and G1 arrest induced by anti-epidermal growth factor receptor monoclonal antibody. Oncogene 2001;20:1913–22.

21. Altieri DC. Survivin and apoptosis control. Adv Cancer Res 2003;88:31–52.

22. Lee CT, Park KH, Adachi Y, et al. Recombinant adenoviruses expressing dominant negative insulin-like growth factor-I receptor demonstrate antitumor effects on lung cancer. Cancer Gene Ther 2003;10:57–63.

23. Lee HY, Moon H, Chun KH, et al. Effects of insulin-like growth factor binding protein-3 and farnesyltransferase inhibitor SCH66336 on Akt expression and apoptosis in non-small-cell lung cancer cells. J Natl Cancer Inst 2004;96:1536–48.

24. Goldstein D, Bushmeyer SM, Witt PL, Jordan VC, Borden EC. Effects of type I and II interferons on cultured human breast cells: interaction with estrogen receptors and with tamoxifen. Cancer Res 1989;49:2698–702.

25. Chun KH, Kosmeder JW II, Sun S, et al. Effects of deguelin on the phosphatidylinositol 3-kinase/Akt pathway and apoptosis in premalignant human bronchial epithelial cells. J Natl Cancer Inst 2003;95:291–302.

26. Parrizas M, Gazit A, Levitzki A, Wertheimer E, LeRoith D. Specific inhibition of insulin-like growth factor-1 and insulin receptor tyrosine kinase activity and biological function by tyrphostins. Endocrinology 1997;138:1427–33.

27. Peterson RT, Desai BN, Hardwick JS, Schreiber SL. Protein phosphatase 2A interacts with the 70-kDa S6 kinase and is activated by inhibition of FKBP12-rapamycinassociated protein. Proc Natl Acad Sci U S A 1999;96:4438–42.

28. Chakravarti A, Loeffler JS, Dyson NJ. Insulin-like growth factor receptor I mediates resistance to anti-epidermal growth factor receptor therapy in primary human glioblastoma cells through continued activation of phosphoinositide 3-kinase signaling. Cancer Res 2002;62:200–7.

29. Balana ME, Labriola L, Salatino M, et al. Activation of ErbB-2 via a hierarchical interaction between ErbB-2 and type I insulin-like growth factor receptor in mammary tumor cells. Oncogene 2001;20:34–47.

30. Gilmore AP, Valentijn AJ, Wang P, et al. Activation of BAD by therapeutic inhibition of epidermal growth factor receptor and transactivation by insulin-like growth factor receptor. J Biol Chem 2002;277:27643–50. Epub 2002 May 13.

31. Ahmad T, Farnie G, Bundred NJ, Anderson NG. The mitogenic action of insulin-like growth factor I in normal human mammary epithelial cells requires the epidermal growth factor receptor tyrosine kinase. J Biol Chem 2004;279:1713–9. Epub 2003 Oct.

32. Kuribayashi A, Kataoka K, Kurabayashi T, Miura M. Evidence that basal activity, but not transactivation, of the epidermal growth factor receptor tyrosine kinase is required for insulin-like growth factor I-induced

activation of extracellular signal-regulated kinase in oral carcinoma cells. Endocrinology 2004;145:4976–84. Epub 2004 Jul 22.
33. Holcik M, Gibson H, Korneluk RG. XIAP: apoptotic brake and promising therapeutic target. Apoptosis 2001; 6:253–61.
34. Gingras AC, Gygi SP, Raught B, et al. Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. Genes Dev 1999;13:1422–37.
35. Herbst RS. Erlotinib (Tarceva): an update on the clinical trial program. Semin Oncol 2003;30:34–46.
36. Amann J, Kalyankrishna S, Massion PP, et al. Aberrant epidermal growth factor receptor signaling and enhanced sensitivity to EGFR inhibitors in lung cancer. Cancer Res 2005;65:226–35.
37. Tracy S, Mukohara T, Hansen M, Meyerson M, Johnson BE, Janne PA. Gefitinib induces apoptosis in the EGFRL858R non-small-cell lung cancer cell line H3255. Cancer Res 2004;64:7241–4.
38. Roskoski R, Jr. The ErbB/HER receptor protein-tyrosine kinases and cancer. Biochem Biophys Res Commun 2004;319:1–11.
39. Garrett TP, McKern NM, Lou M, et al. Crystal structure of the first three domains of the type-1 insulin-like growth factor receptor. Nature 1998;394: 395–9.
40. Jimeno A, Rubio-Viqueira B, Amador ML, et al. Epidermal growth factor receptor dynamics influences response to epidermal growth factor receptor targeted agents. Cancer Res 2005;65:3003–10.
41. Tan AR, Yang X, Hewitt SM, et al. Evaluation of biologic end points and pharmacokinetics in patients with metastatic breast cancer after treatment with erlotinib, an epidermal growth factor receptor tyrosine kinase inhibitor. J Clin Oncol 2004;22:3080–90.
42. Gregorc V, Ceresoli GL, Floriani I, et al. Effects of gefitinib on serum epidermal growth factor receptor and HER2 in patients with advanced non-small cell lung cancer. Clin Cancer Res 2004;10:6006–12.
43. Rao RD, Mladek AC, Lamont JD, et al. Disruption of parallel and converging signaling pathways contributes to the synergistic antitumor effects of simultaneous mTOR and EGFR inhibition in GBM cells. Neoplasia 2005;7:921–9.
44. Gschwind A, Zwick E, Prenzel N, Leserer M, Ullrich A. Cell communication networks: epidermal growth factor receptor transactivation as the paradigm for interreceptor signal transmission. Oncogene 2001;20: 1594–600.
45. Roudabush FL, Pierce KL, Maudsley S, Khan KD, Luttrell LM. Transactivation of the EGF receptor mediates IGF-1-stimulated shc phosphorylation and ERK1/2 activation in COS-7 cells. J Biol Chem 2000; 275:22583–9.
46. Pierce KL, Luttrell LM, Lefkowitz RJ. New mechanisms in heptahelical receptor signaling to mitogen activated protein kinase cascades. Oncogene 2001;20: 1532–9.
47. Marinissen MJ, Gutkind JS. G-protein-coupled receptors and signaling networks: emerging paradigms. Trends Pharmacol Sci 2001;22:368–76.
48. Pollak M. Insulin-like growth factor physiology and cancer risk. Eur J Cancer 2000;36:1224–8.
49. Merrill MJ, Edwards NA. Insulin-like growth factor-I receptors in human glial tumors. J Clin Endocrinol Metab 1990;71:199–209.

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Correlative analyses of plasma cytokine / angiogenic factor (C/AF) profile, gender and outcome in a randomized, three-arm, phase II trial of 1st-line vandetanib (VAN) and / or carboplatin plus paclitaxel (CP) for advanced non small cell lung cancer (NSCLC).

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Background: VAN (ZD6474) is an oral inhibitor of VEGFR, EGFR and RET. In a phase II trial, 181 patients with advanced NSCLC were randomized to 1st-line treatment with VAN, CP or VAN + CP. Progression free survival (PFS) was prolonged for VAN + CP vs CP (Heymach et al, submitted for ASCO 2007). Exploratory subgroup analyses suggest gender differences in PFS benefit for VAN + CP vs CP (HR 0.47 in females vs 1.05 in males). We performed exploratory analyses of plasma levels of 35 C/AFs to investigate gender differences and potential prognostic or predictive markers.
Methods: Plasma was collected at baseline (n = 123; VAN 55, CP 32, VAN + CP 36), day (D) 8 (n = 104), D22 (n = 95), and D43 (n = 83). We used multiplex bead assays to measure 33 plasma C/AFs, including VEGF, basic FGF, EGF, HGF, E-selectin, ICAM-1, MMP-9, multiple chemokines and interleukins (IL). Osteopontin and sVEGFR-2 were measured by ELISA. Cox models were applied on PFS to identify prognostic / predictive markers after rank transformation and adjusted for covariates.
Results: Significant gender differences in baseline C/AF levels were seen for IL-15, IL-1RA, IL-2R, MIG, and MIP-1A (all higher in females, all $p \leq .022$). Controlling for gender and treatment, high baseline E-selectin ($p = .01$), IL-6 ($p = .018$), and IL-2R ($p = .008$) were adverse prognostic indicators for PFS. Controlling for gender, the tests for treatment by factor interactions (to assess whether treatment effect was different in patients with low and high C/AF levels) were significant for baseline HGF ($p = .04$) and IL-2R ($p = .008$). For both HGF and IL-2R, low levels were associated with prolonged PFS in the VAN arm, but were not associated with differences in the CP or VAN + CP arms. Significant changes in VEGF (rise) and sVEGFR-2 (decrease) occurred with treatment in the VAN arm; IL12, IL-1RA, MMP-9 and MCP-1 changed in the CP and VAN + CP arms.
Conclusions: There are gender differences in PFS benefit from VAN and in plasma C/AF profile. Several C/AFs were of prognostic value, whereas low HGF and IL-2R were predictive of benefit in the VAN but not CP and VAN + CP arms. These gender differences and markers warrant further investigation.

Cancer

OR02-1

IGF-1R/EGFR HETERODIMERIZATION AND RESISTANCE TO EPIDERMAL GROWTH FACTOR RECEPTOR TYROSINE KINASE INHIBITORS IN NON-SMALL-CELL LUNG CANCER

Ho-Young Lee*, Floriana Morgillo, Waun K Hong. *UT, M.D. Anderson Cancer Center, USA*

Background: Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) have been used to treat lung cancers, but resistance to these agents has been observed. This study was designed to investigate whether the insulin-like growth factor (IGF)-mediated signaling pathway induces resistance to the EGFR TKIs in lung cancer. Methods: The antitumor activities and action mechanisms of EGFR inhibitors (erlotinib, gefinitib, cetuximab), single or in combination with IGF-IR inhibitors, were assessed in vitro in a subset of non-small-cell lung cancer (NSCLC) cell lines by the MTT assay, flow cytometry-based TUNEL assay, soft agar, confocal microscopy, metabolic labeling, coimmunoprecipitation, and northern and western blot analyses, and in vivo in animal models. Correlation of EGFR and IGF-1R expression was assessed using lung tissues from patients with NSCLC. Results: EGFR TKI inhibited the proliferation and anchorage-dependent and -independent colony-forming ability of NSCLC cells, which induced apoptosis, only when IGF-1R signaling was blocked. Treatment with EGFR TKIs, but not with the EGFR antibody, induced EGFR:IGF-1R heterodimerization on cell surface and activation of the IGF-1R, resulting in the stimulation of PI3K/Akt/mTOR pathway, promoting the de novo protein biosynthesis of survivin and EGFR, resulting in the survival of NSCLC cells. When IGF-IR and mTOR were blocked, treatment of EGFR-TKIs induced apoptosis in NSCLC cells, resulting in suppression of the NSCLC tumor growth. When we tested the expression of IGF-R and EGFR in human lung tissue, 9/14 tumor samples exhibited increased expression of EGFR and 7/9 samples showed a correlative increases in IGF-IR protein levels compared to their paired normal counterparts. These findings suggest that simultaneous targeting of EGFR and IGF-1R signaling pathways might be an effective therapeutic strategy against NSCLC.

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An Application of Adaptive Randomization Using Hierarchical Bayes Model in a Prospective Biomarker-Based Clinical Trial

Suyu Liu, Edward Kim, Xian Zhou, Ignacio Wistuba, Roy Herbst, Jeffrey Lewis, J. Jack Lee

Abstract

To identify best-matched treatments for patients, we implement an outcome-based adaptive randomization in BATTLE trial (Biomarker-based Approaches of Targeted Therapy for Lung Cancer Elimination). It consists of 4 parallel phase II studies each with targeted therapies. Patients require a core biomarker biopsy prior to randomization. A hierarchical Bayes model is used to characterize efficacy rates among the 4 treatments for each biomarker profile. Based on the posterior clinical efficacy, patients are adaptively randomized according to their real-time biomarker status. The operating characteristics based on simulations indicate that the design can accurately identify the effective biomarker-treatment combinations, and allocates more patients to more efficacious treatments – a step toward "personalizing medicine". Examples of data realization and practical considerations will be given.

Key words: adaptive randomization, clinical trial, hierarchical Bayes model, operating characteristics

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428 POSTER

Induction of survivin expression via activation of insulin-like growth factor-1 receptor/epidermal growth factor receptor heterodimer: a novel resistance mechanism of EGFR tyrosine kinase inhibitors in non-small cell lung cancer

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Background: The role of EGFR signaling pathway in tumor progression has long been appreciated. However, the overall response rate to EGFR tyrosine kinases (TKIs) is low and the causes of resistance to these drugs are poorly defined. This study was designed to investigate the mechanisms mediating resistance to the drugs.
Methods: The antitumor activities and action mechanisms of EGFR inhibitors (erlotinib, gefinitib, cetuximab), single or in combination with Insulin-like growth factor-1 receptor (IGF-IR) inhibitors, were assessed *in vitro* in a subset of non-small-cell lung cancer (NSCLC) cell lines by the MTT assay, flow cytometry-based TUNEL assay, anchorage-dependent and -independent colony formation, metabolic labeling, coimmunoprecipitation, and northern and western blot analyses and *in vivo* in animal models. EGFR and IGF-1R expression was assessed in lung tissue samples from patients with NSCLC.
Results: EGFR TKIs inhibited the proliferation and anchorage-dependent and -independent colony-forming abilities of NSCLC cells by inducing apoptosis only when IGF-1R signaling was blocked. Treatment with EGFR TKIs, but not with the EGFR antibody, induced EGFR:IGF-1R heterodimerization on cell membrane and activation of the IGF-1R, resulting in the stimulation of PI3K/Akt/mammalian target of rapamycin (mTOR) pathway, promoting the *de novo* protein synthesis of survivin and EGFR, resulting in the survival of NSCLC cells. Inhibition of IGF-1R activation, suppression of mTOR-mediated protein synthesis, or knockdown of survivin expression abolished resistance to the EGFR TKIs and induced apoptosis in NSCLC cells *in vitro* and *in vivo*. The majority of patients with EGFR-overexpressing NSCLC had correlatively high levels of IGF-1R in tumors compared with those in normal counterparts.
Conclusions: IGF-1R activation interferes with the antitumor activity of EGFR TKIs and IGF-1R expression may serve as a predictor for EGFR TKI resistance in NSCLC. IGF-1R-targeting combination treatment is required when EGFR TKIs are considered as therapeutic strategies for NSCLC patients.

Presence of amphiregulin autocrine-loop predicts in vitro sensitivity of *EGFR* wild type NSCLC and HNSCC cell lines to gefitinib and cetuximab

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Lowe Center for Thoracic Oncology and Department of Medical Oncology
Dana-Farber Cancer Institute and Departments of Medicine, Brigham and Women's Hospital and Harvard Medical School

Background

Epidermal growth factor receptor (EGFR) is a therapeutic target in non-small cell lung cancer (NSCLC) and head and neck squamous cell carcinoma (HNSCC). Patients with NSCLC with activating mutations in *EGFR* often have significant tumor regressions with gefitinib treatment. In addition, approximately 30% of patients treated with gefitinib develop stable disease implicating additional mechanisms of gefitinib sensitivity. The majority of such patients do not harbor *EGFR* mutations and *EGFR* mutations in HNSCC are rare. As EGFR can be activated by one of its ligands (amphiregulin, EGF, TGFα), we examined the relationship of EGFR ligands to the efficacy of gefitinib and cetuximab using in vitro growth assays of *EGFR* wild type NSCLC and HNSCC cell lines.

Methods

Amphiregulin, EGF and TGF-alpha in cell culture media was analyzed with ELISA assays in 18 cell lines, 14 NSCLC and 4 HNSCC cell lines. This included four NSCLC with *EGFR* mutation. The remaining 14 cell lines were wild type for *EGFR*. All had wild type *KRAS* except for H358. The in vitro sensitivity was analyzed using MTS assay. EGFR, HER3, Akt, ERK1/2, Cyclin D1 and phosphorylation of these proteins were analyzed with Western blotting. Cell cycle was analyzed by flow cytometry.

Result

TGF-alpha (range, 0 to 4.8pg/ml) was only detected in Calu3 (116.6pg/ml) and H1666 (4.8pg/ml) cells. EGF (range, 0 to 2.2pg/ml) was detected at negligible level and did not vary significantly. The presence of amphiregulin varied significantly among the cell lines (range, 4.6 to 1625.8pg/ml). All *EGFR* mutant cell lines had negligible levels of amphiregulin (<50.3pg/ml). Seven cell lines of the 14 cell lines with wild-type EGFR had > 250 pg/ml (range, 718.4 to 1625.8pg/ml) of amphiregulin detectable in media and all

were sensitive to gefitinib with an IC50 of < 1 μM (range, 0.10 to 0.33). In addition, they were also sensitive to cetuximab (mean growth inhibition following10ug/ml cetuximab 56.8% (range, 33.1 to 87.1 %)) while those producing < 250 pg/ml were resistant (mean growth inhibition following10ug/ml cetuximab 0.95% (range –4.4 to 15.3%)). Cell lines producing < 250 pg/ml of amphiregulin were also resistant to gefitinib in vitro (IC > 1 μM). In addition 6/7 sensitive cell lines expressed p-ERBB3 in contrast to 1/7 resistant cell lines. The amphiregulin producing cell lines underwent G1/S arrest following either gefitinib or cetuximab treatment. Furthermore, amphiregulin neutralizing antibodies inhibited growth of 3 amphiregulin producing cell lines but not HN28, which did not produce amphiregulin. Amphiregulin producing sensitive cell lines exhibited dose dependent decrease in p-EGFR, p-ERBB3, p-Akt, p-ERK 1/2 and cyclin D1 following gefitinib. Cetuximab led to a dose dependent decrease in p-EGFR, p-ERK 1/2 and cyclin D1. Gefitinib and cetuximab had no effect on these proteins in cells without amphiregulin.

Conclusion

Our findings suggest that in *EGFR* wild type NSCLC and HNSCC cell lines the presence of an amphiregulin autocrine loop is a major determinant of in vitro sensitivity to gefitinib and cetuximab. In addition to *EGFR* mutations, amphiregulin expression may be suitable biomarker to select patients likely to benefit from gefitinib treatment.

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A clinical trial design applying Bayesian adaptive randomization for targeted therapy development in lung cancer - A step toward personalized medicine

Author Block: X. Zhou[1], E. S. Kim[2], R. S. Herbst[2], S. Liu[3], I. I. Wistuba[4], L. Mao[2], J. Lewis[3], S. M. Lippman[2], W. K. Hong[2], J. J. Lee[3];
[1]Genentech, South San Francisco, CA, [2]Thoracic, Head/Neck Medical Oncology, M. D. Anderson Cancer Center, Houston, TX, [3]Biostatistics, M. D. Anderson Cancer Center, Houston, TX, [4]Pathology, M. D. Anderson Cancer Center, Houston, TX.

Abstract:
Background:
Multiple axes of signaling pathways are associated with lung carcinogenesis. These signaling axes are different in pts (pts) and their cancers. Utilization of molecularly targeted agents may inhibit these specific aberrant pathways and lead to clinical efficacy. Biomarkers expressions can be used as indicators for the aberrant signaling to identify effective targeted therapy.

Methods:
The “BATTLE” program, “Biomarker-integrated Approaches of Targeted Therapy of Lung Cancer Elimination,” consists of an umbrella screening trial and 4 parallel phase II targeted therapies trials (with erlotinib, sorafenib, vandetanib, and the combination of erlotinib and bexarotene) in advanced non-small cell lung cancer pts with prior chemotherapy. All pts will have biopsy samples taken for biomarker profile assessment prior to the randomization. A "surrogate response" to treatment is defined as progression free at 8 weeks after randomization. The Bayesian ordinal probit model is used to characterize the response rate. Pts with certain biomarker profile will be adaptively randomized (AR) to one of the 4 treatment arms with the randomization rate based on the updated response rate based on accumulated data in the trial. For each biomarker profile, better performing arms will have higher randomization rates and vise versa. Early stopping rules are set so that low-performing arms may be suspended for new patient entry.

Results:
Based on extensive simulation studies, the proposed design with a total of 200 pts has desirable operating characteristics to: (1) identify effective agents with high probability; (2) suspend ineffective agents; and (3) treat more pts with effective agents according to their biomarker profiles. The Bayesian design incorporates prior data and findings from the current pts to form better estimates of the treatment efficacy for pts with different

biomarker profiles. The design continues to “learn” and improve the estimates as the trial moves along.

Conclusion:
The Bayesian AR design is a smart and ethical design and ideally suitable for the development of targeted therapy. It may help in identifying effective agents based on pts’ tumor biomarker profile and thus treat more pts with effective therapies.

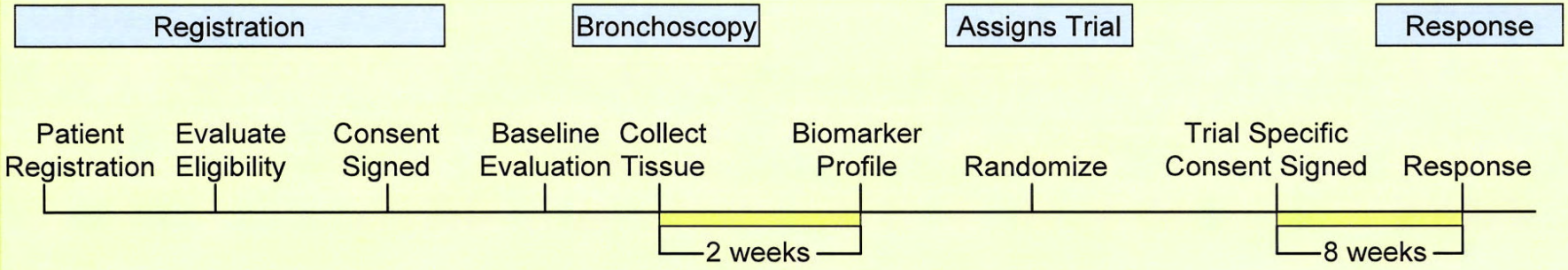
APPENDIX B

Biostatistics Core

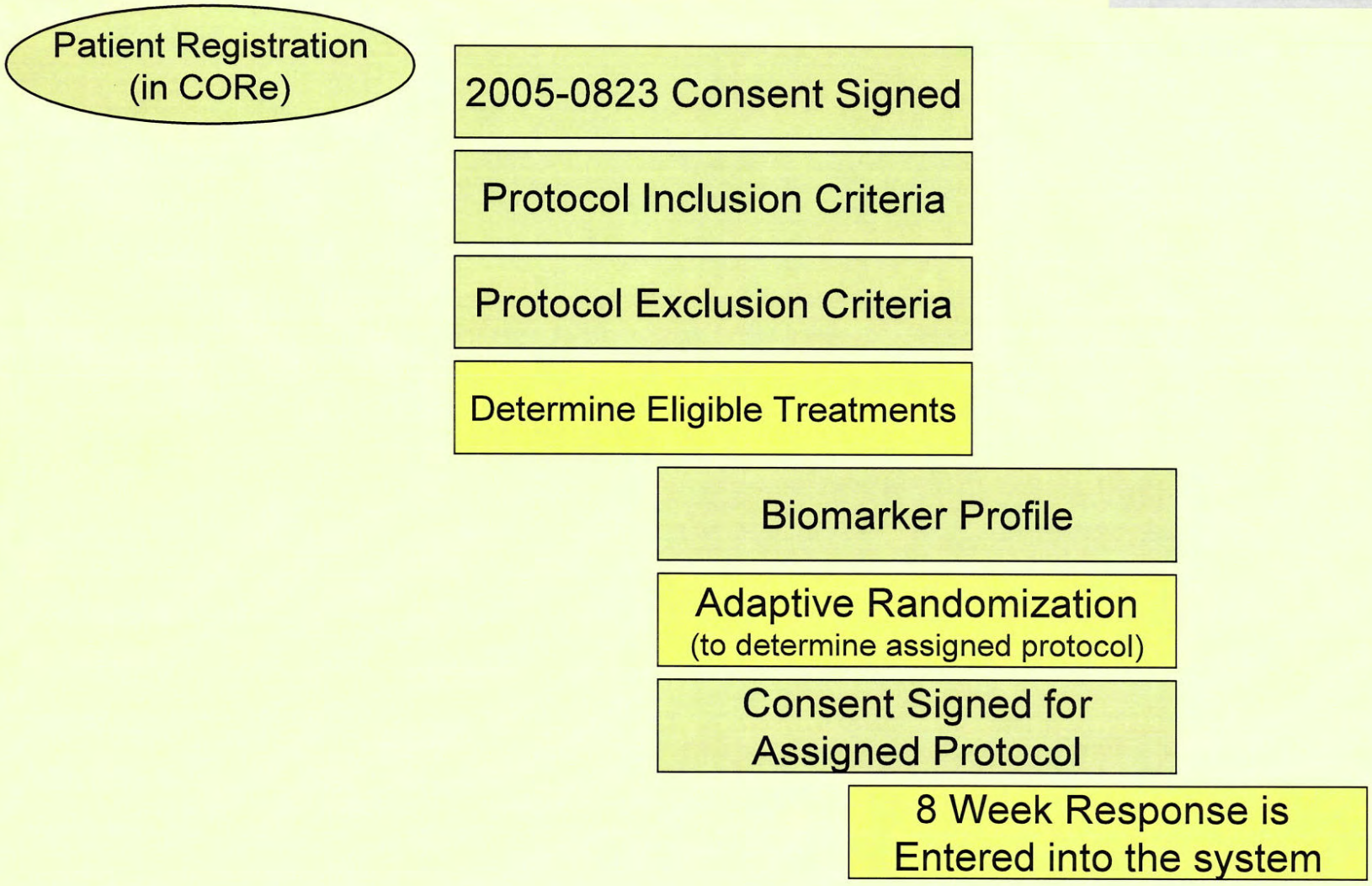
2005-0823 BATTLE

Randomization Workflow Process

Battle Workflow Timeline



BATTLE Workflow



BATTLE Workflow

Patient Registration
(in CORe)

- Patients are initially entered into CORe for registration
- Patients relevant information is transferred into the BATTLE database

BATTLE Workflow

- 2005-0823 Consent Signed
- Protocol Inclusion Criteria
- Protocol Exclusion Criteria
- Determine Eligible Treatments

Inclusion criteria and Exclusion criteria questions are answered to determine eligible treatments that the patient qualifies for.

Protocol	Treatment
1) 2005-0824	Tarceva
2) 2005-0825	ZD6474
3) 2005-0826	Tarceva and Targretin
4) 2005-0827	Sorafenib

BATTLE Workflow

Biomarker Profile

- Tissue Sample is collected by the bronchoscopy at baseline visit
- Biomarker Core will complete Biomarker Profile in two weeks
- Biomarker information is entered into the BATTLE database
- Positive or Negative Biomarker results are use to determine the marker group used for Adaptive Randomization

Biomarker Group
1) EGFR (+)
2) K-ras (+) or B-raf (+)
3) VEGF (+) or VEGFR (+)
4) RXRs (+) or Cyclin D1 (+)
5) If all (-)

BATTLE Workflow

Adaptive Randomization
(to determine assigned protocol)

Protocol	Treatment
1) 2005-0824	Tarceva
2) 2005-0825	ZD6474
3) 2005-0826	Tarceva and Targretin
4) 2005-0827	Sorafenib

Biomarker Group
1) EGFR (+)
2) K-ras (+) or B-raf (+)
3) VEGF (+) or VEGFR (+)
4) RXRs (+) or Cyclin D1 (+)
5) If all (-)

Consent Signed for
Assigned Protocol

- Eligible treatments and the Biomarker Group are used in the Adaptive Randomization algorithm to assign the patient to the protocol.

- Another consent is signed for the assigned protocol.

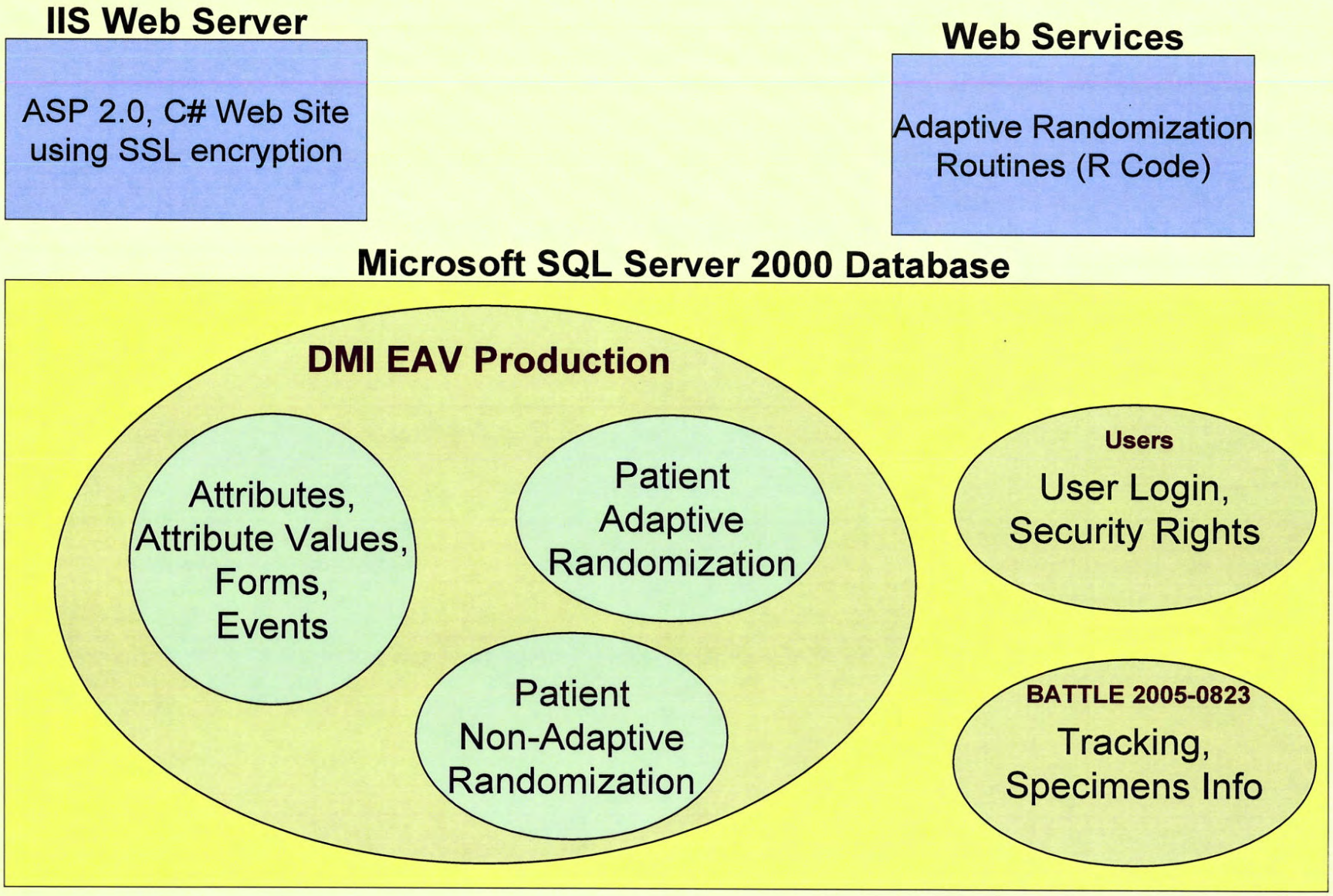
BATTLE Workflow

8 Week Response is Entered into the system

- After eight weeks, the patients response to the treatment is entered into the database.

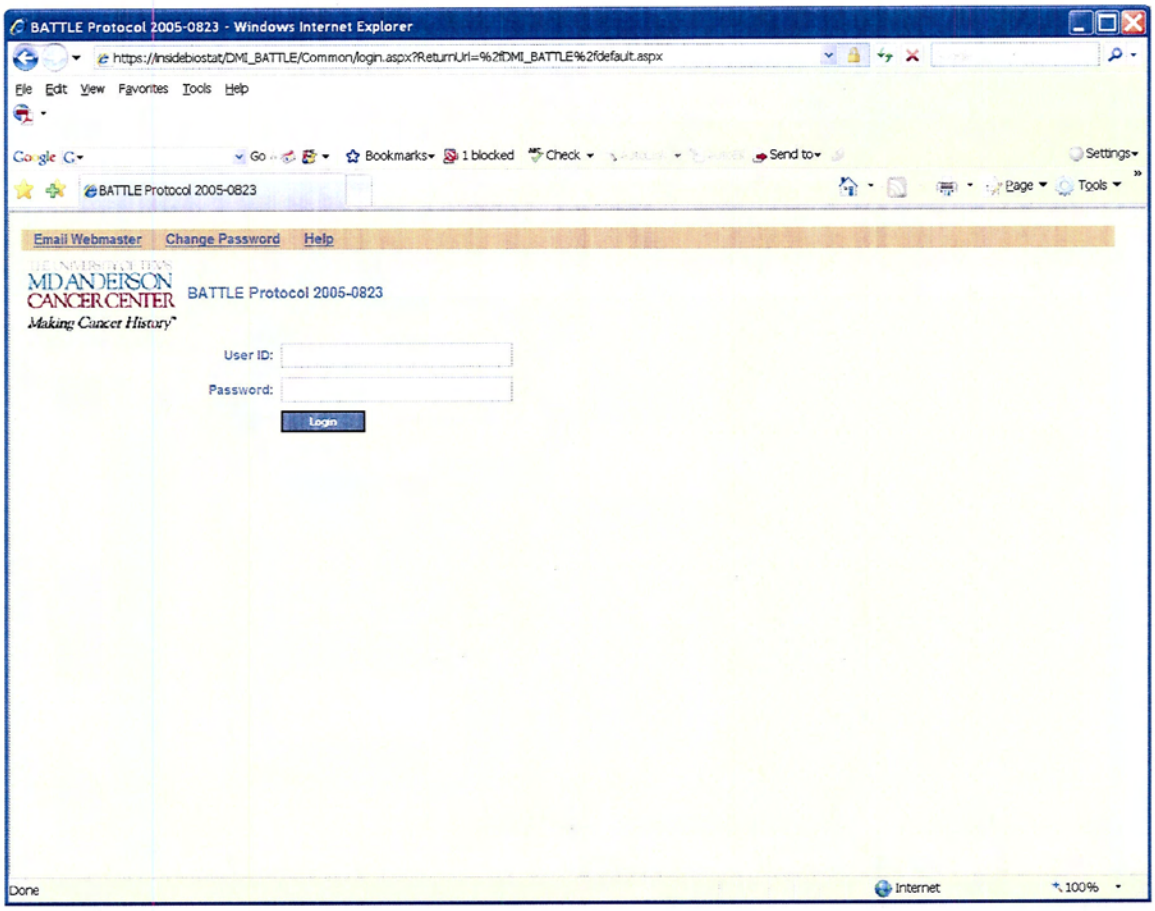
- This information will be used for the Adaptive Randomization.

BATTLE Database overview

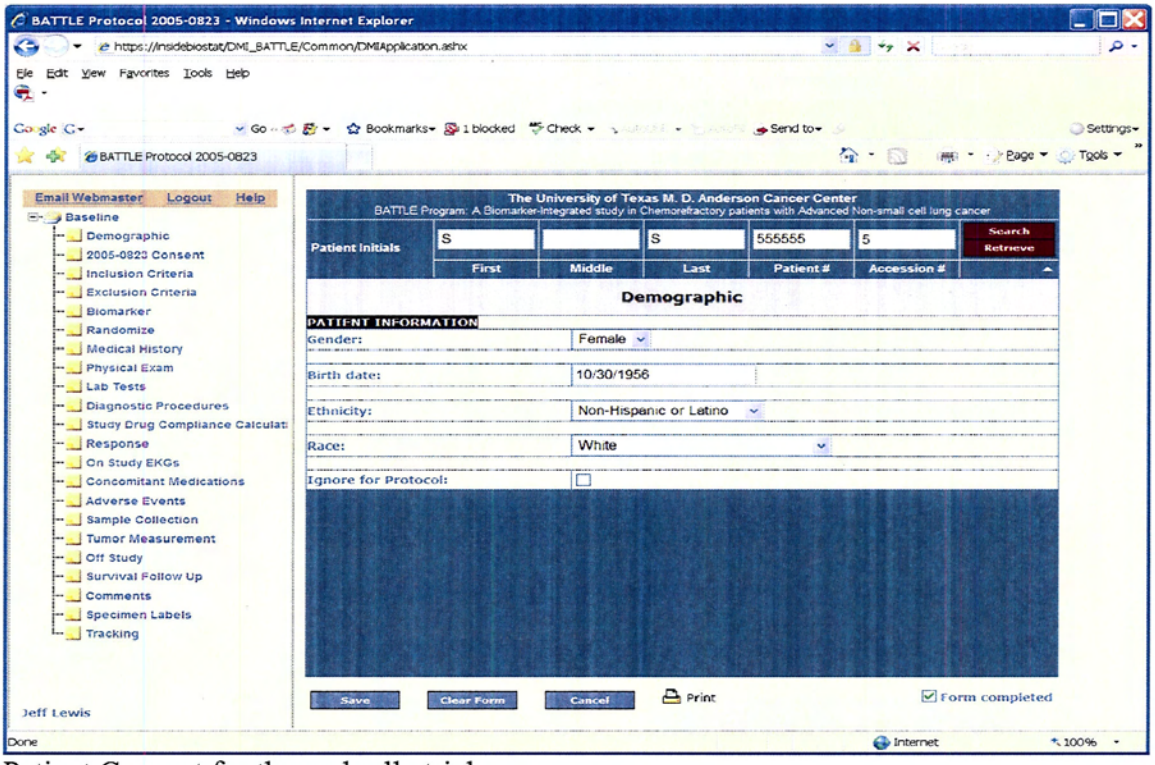


BATTLE Login screen to ensure only authorized users can access the system.

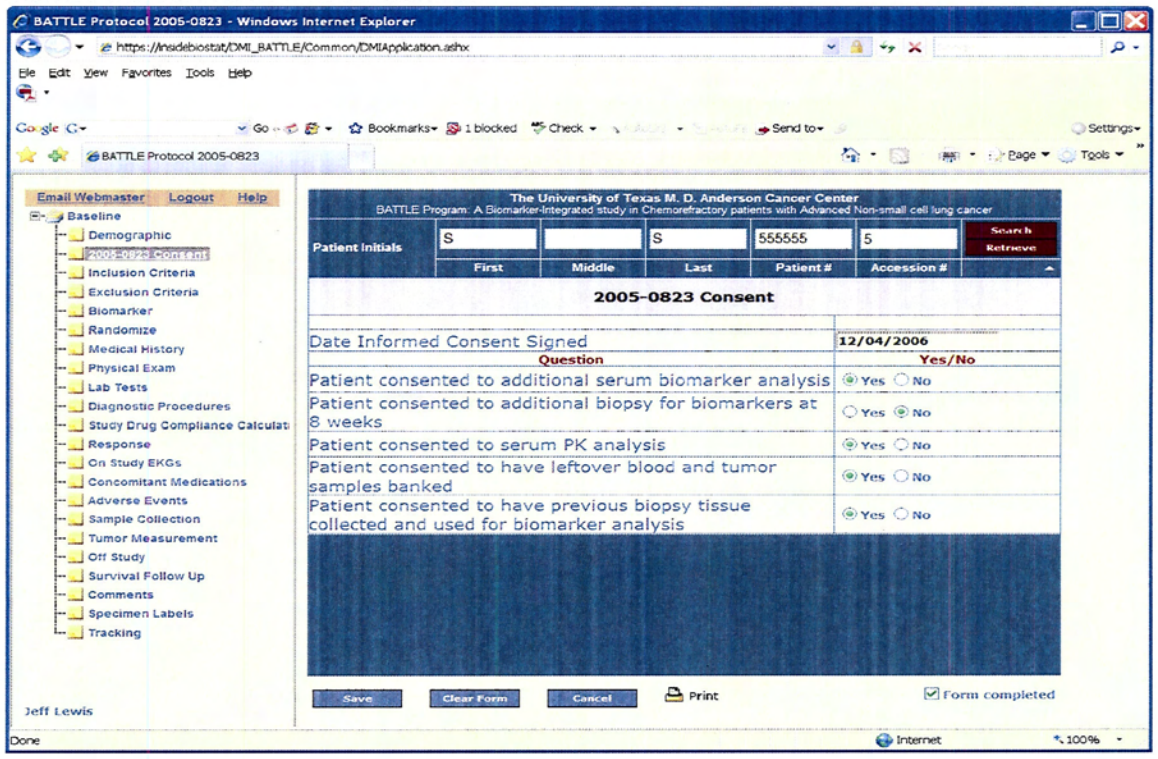
NOTE: All screen shots were created using fictitious data to show the functionality of the data system and, in the meantime, to protect patient confidentiality.



Patient demographic information

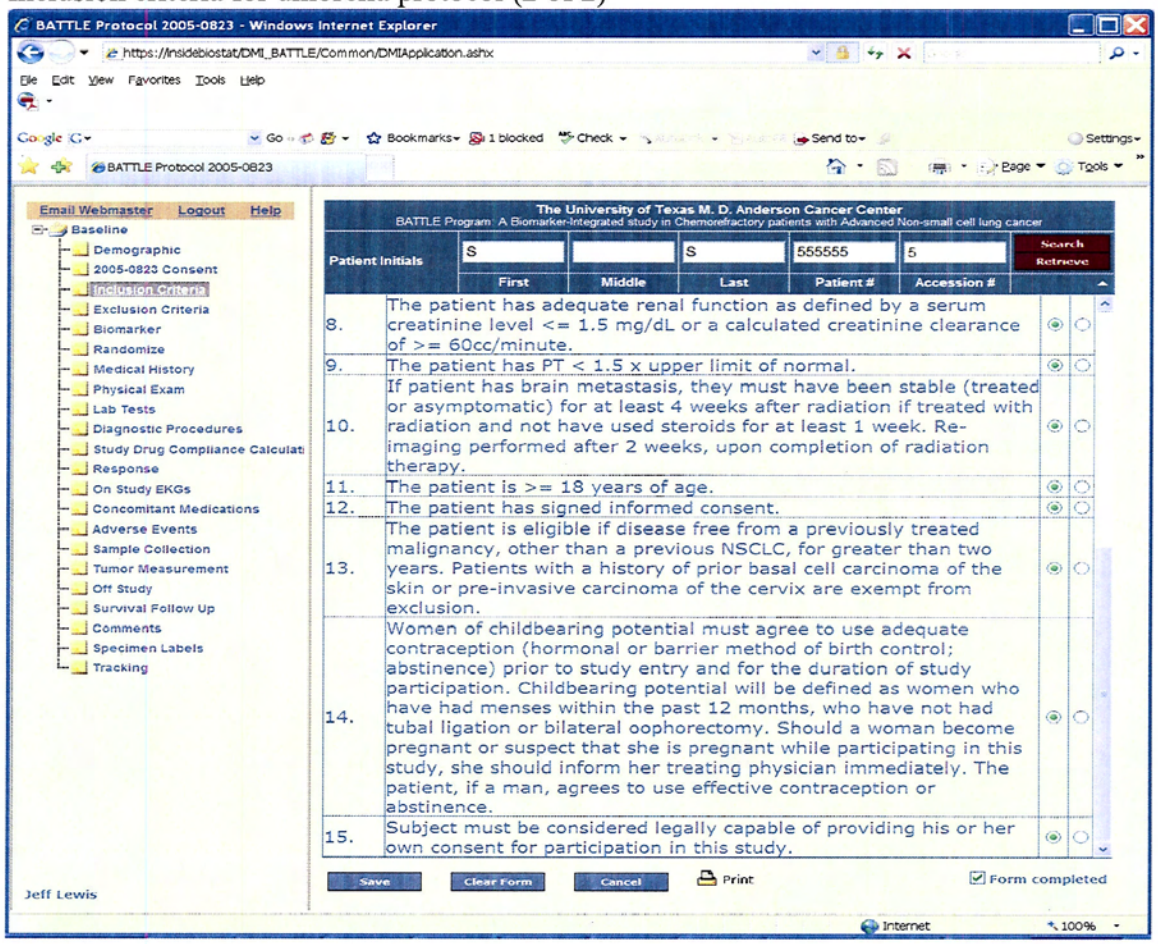


Patient Consent for the umbrella trial

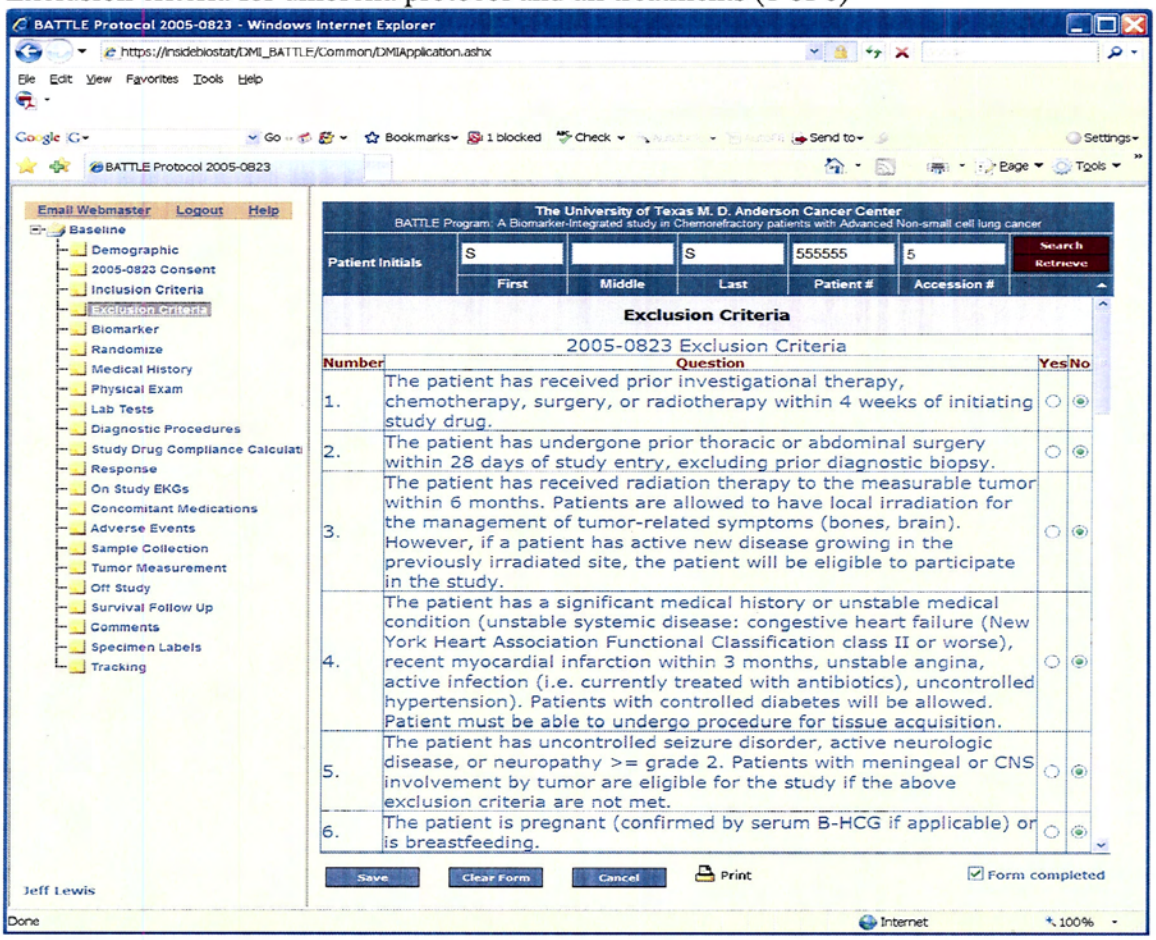


Inclusion criteria for umbrella protocol (1 of 2)

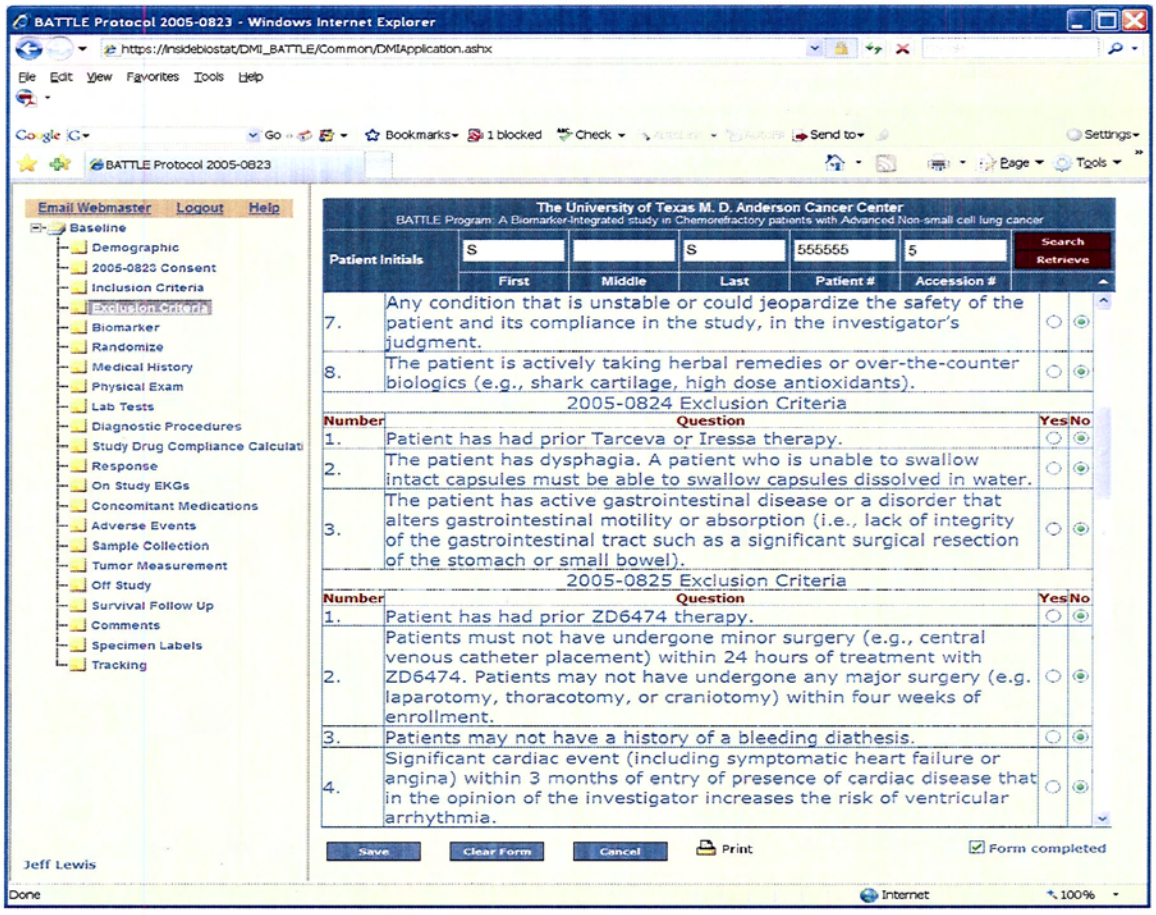
Inclusion criteria for umbrella protocol (2 of 2)



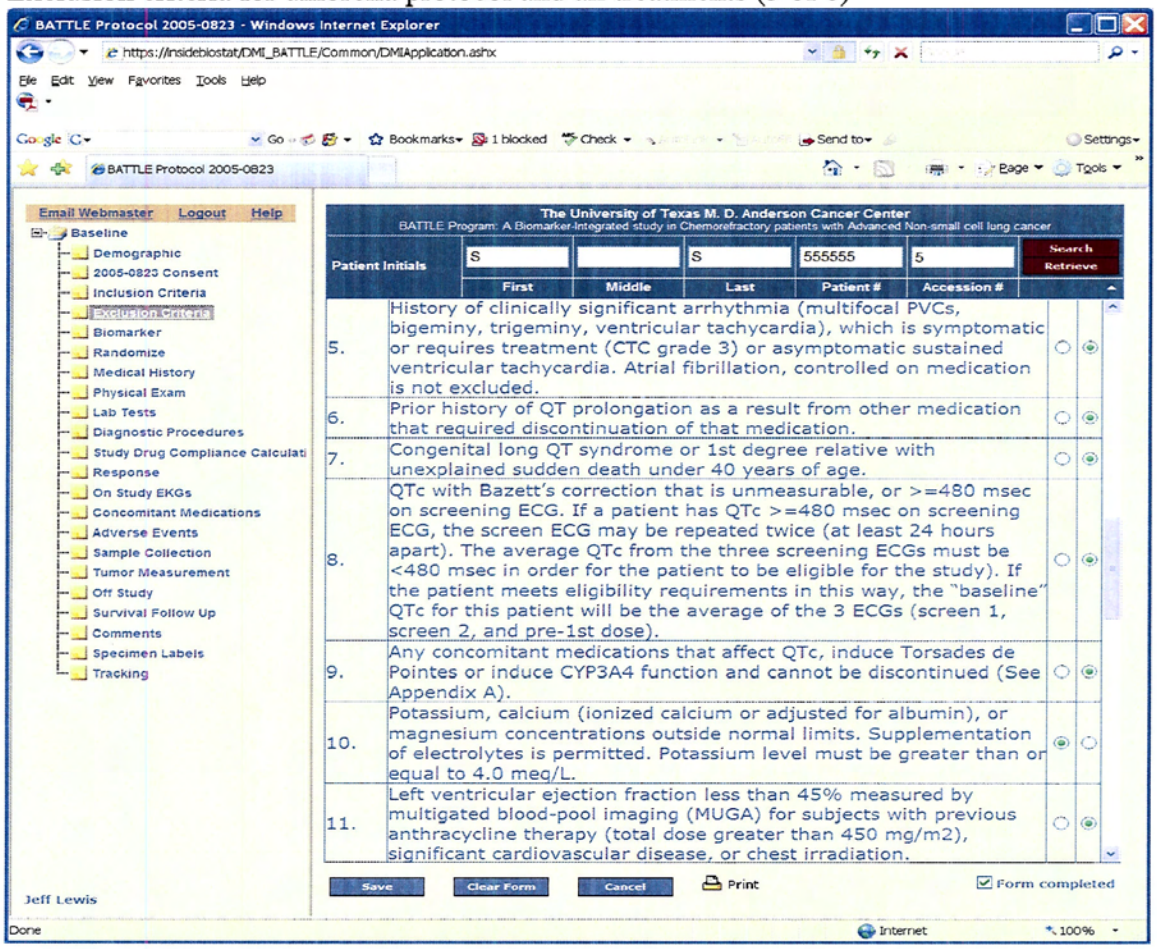
Exclusion criteria for umbrella protocol and all treatments (1 of 6)



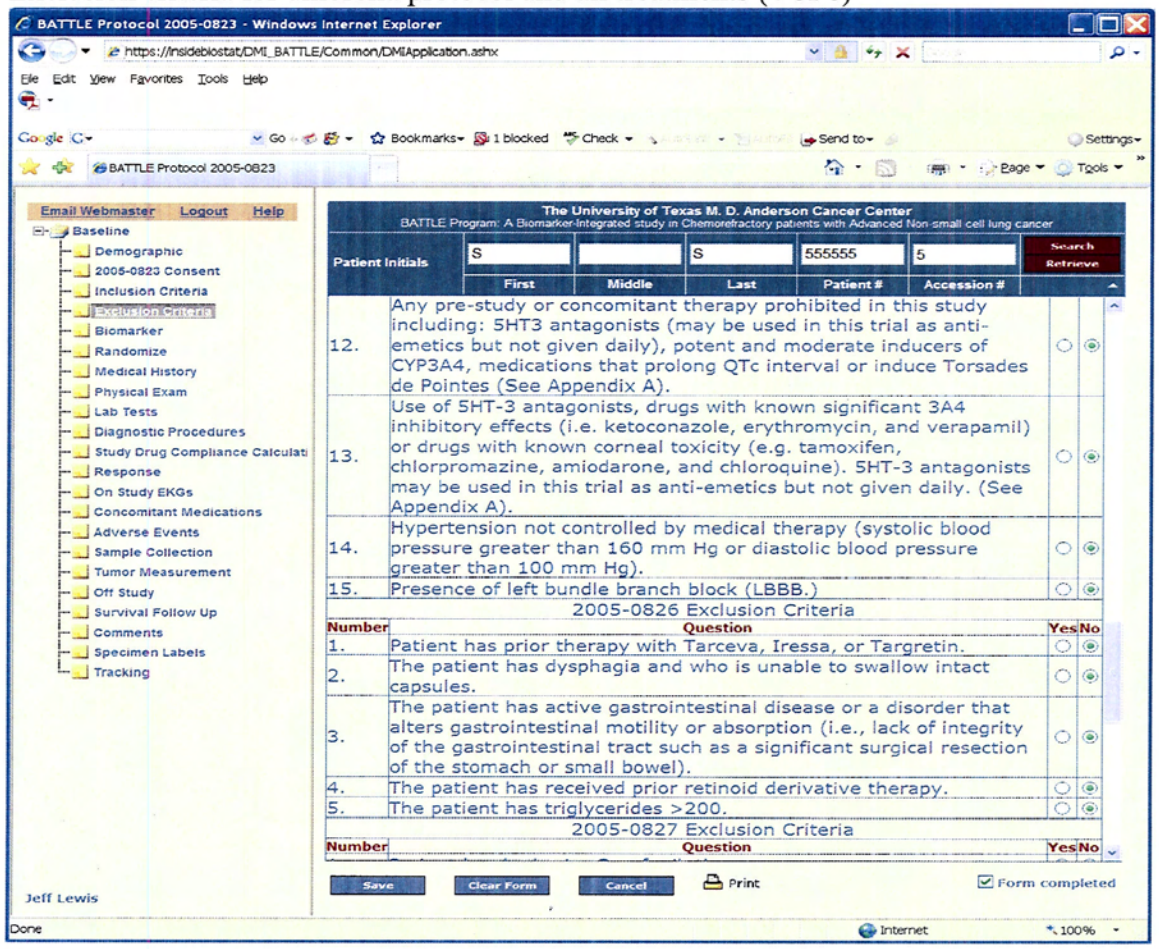
Exclusion criteria for umbrella protocol and all treatments (2 of 6)



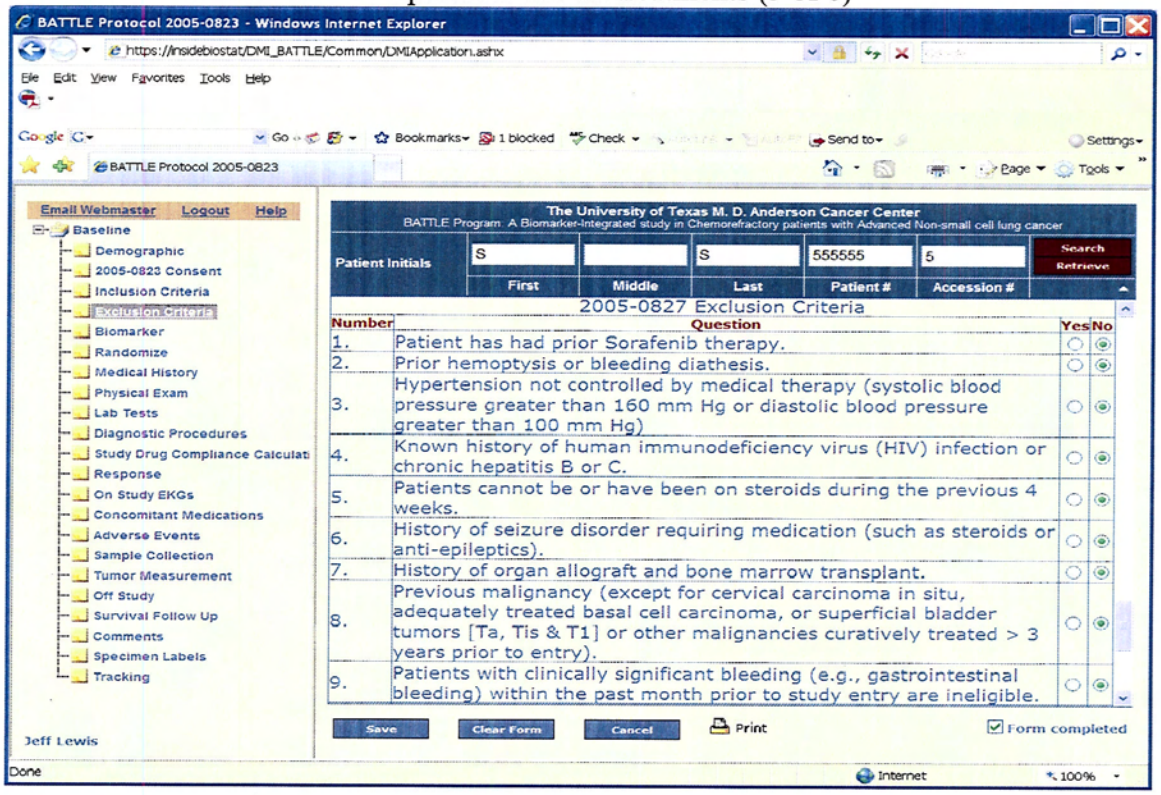
Exclusion criteria for umbrella protocol and all treatments (3 of 6)



Exclusion criteria for umbrella protocol and all treatments (4 of 6)

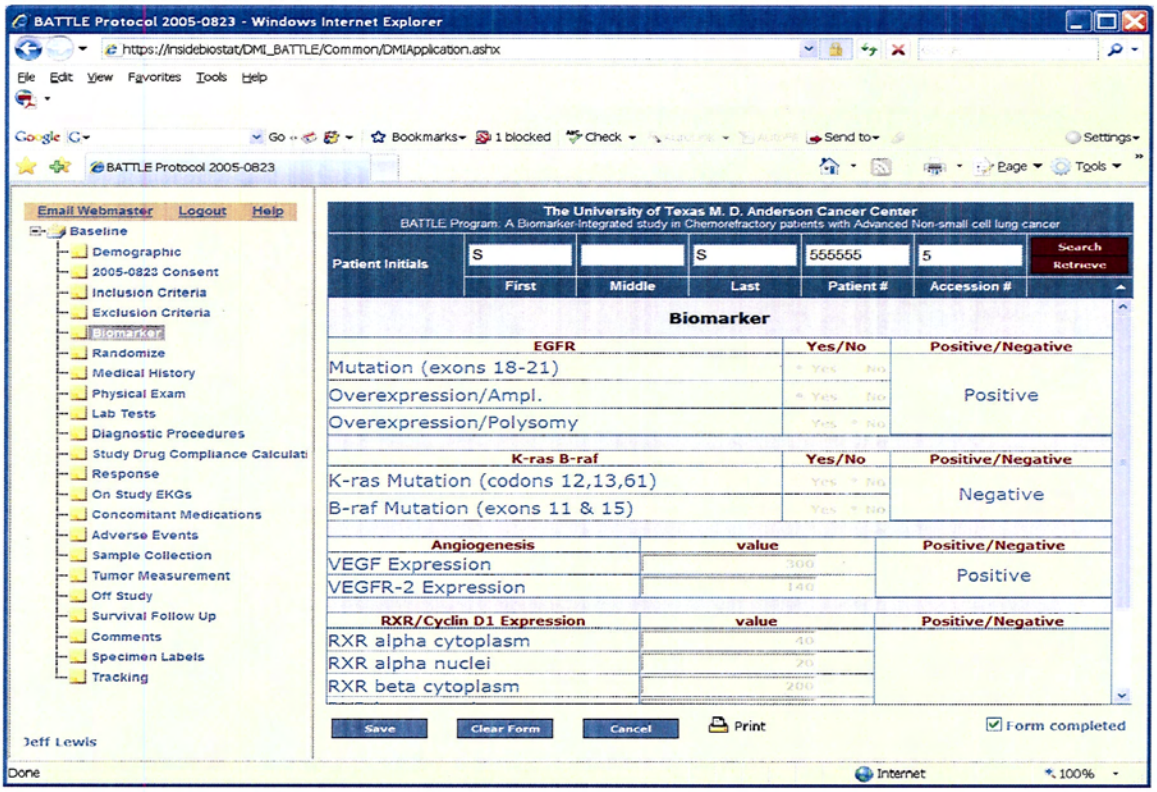


Exclusion criteria for umbrella protocol and all treatments (5 of 6)

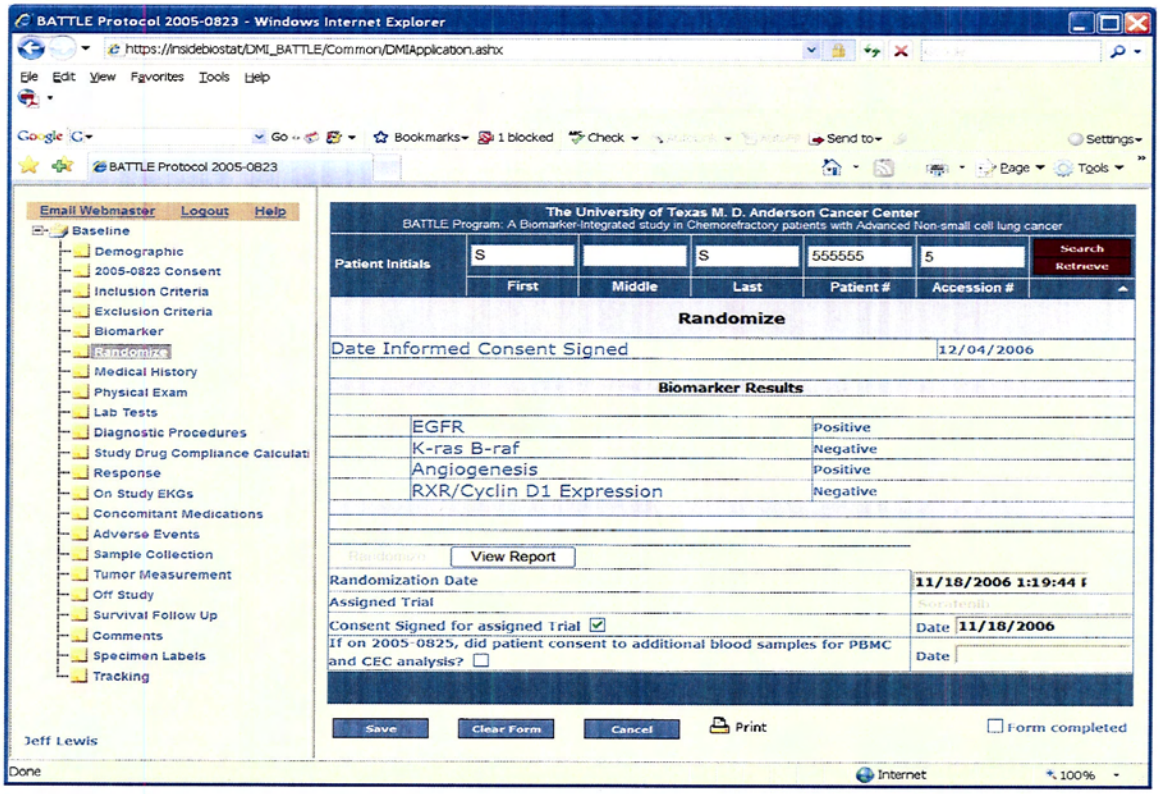


Exclusion criteria for umbrella protocol and all treatments (6 of 6)

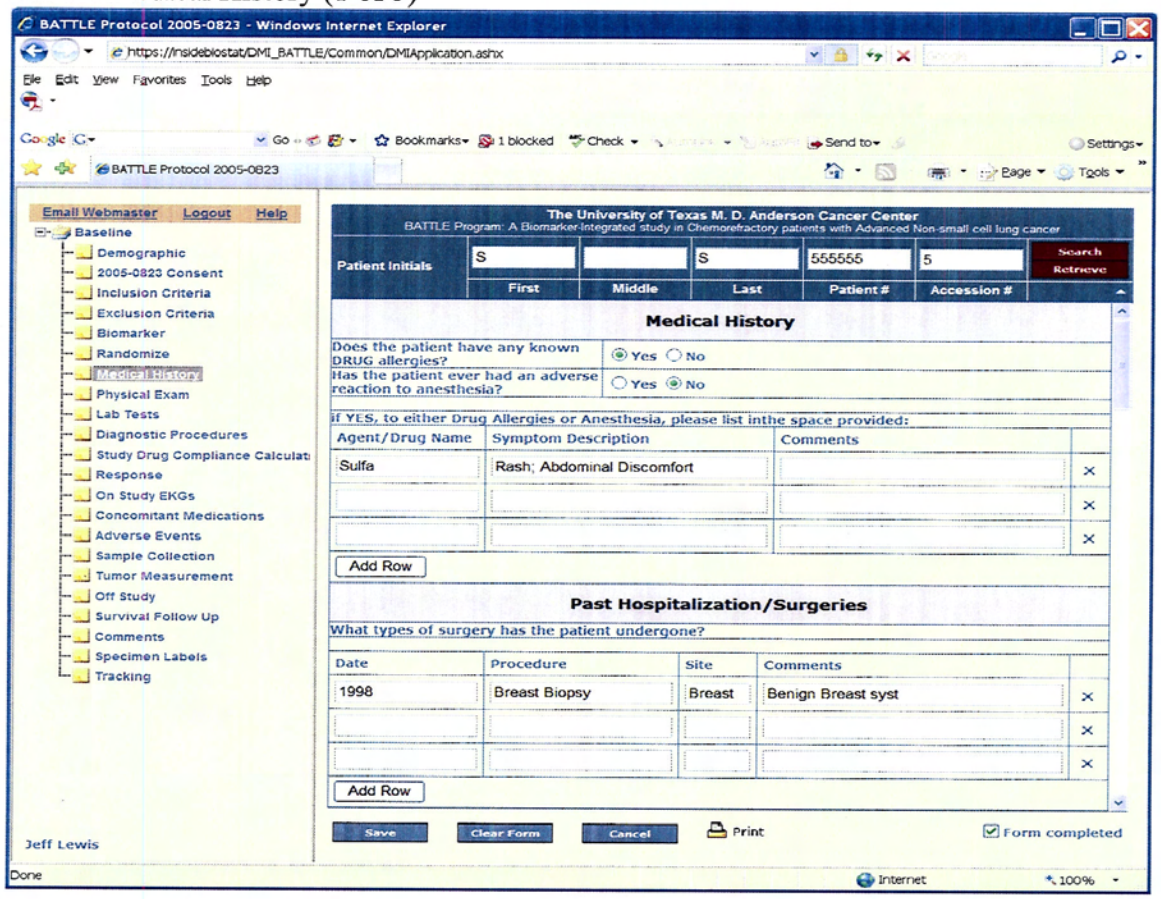
Biomarker information



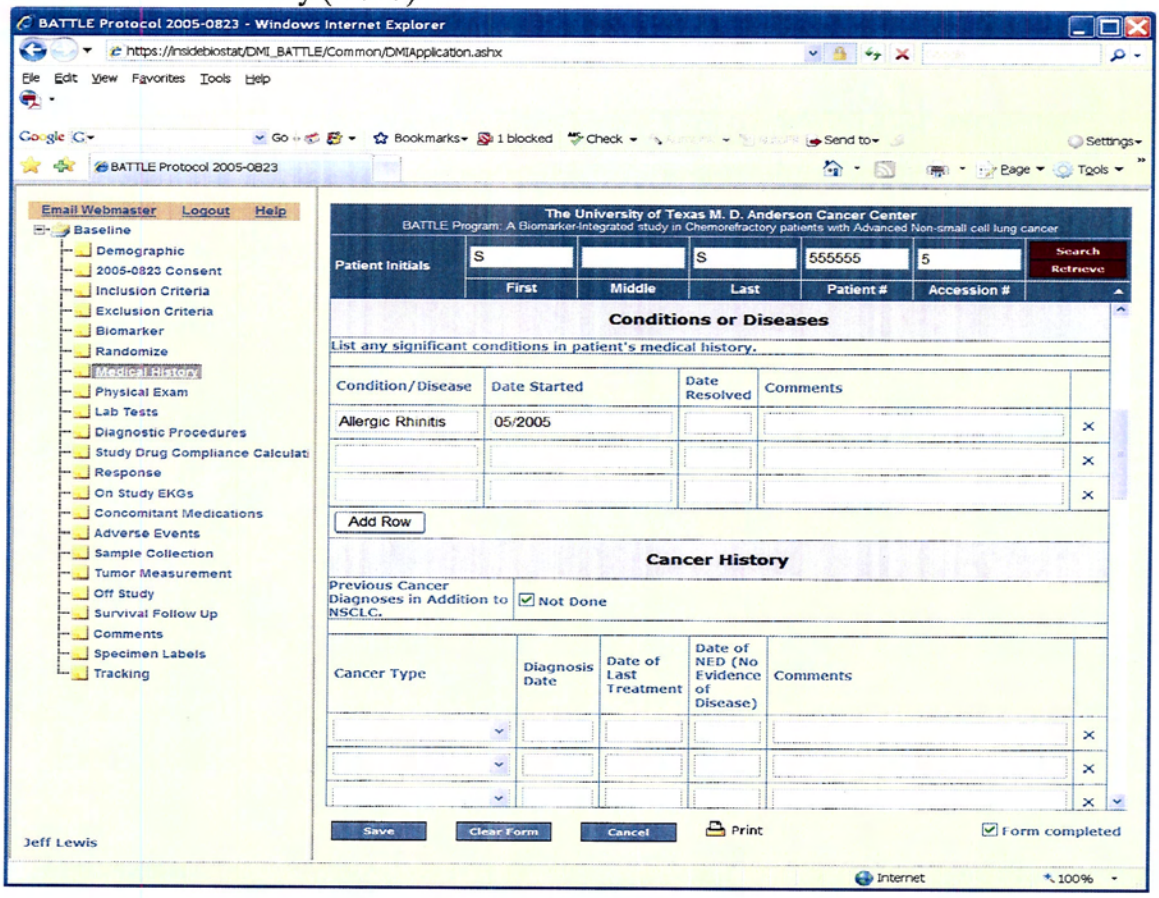
Randomization for treatment



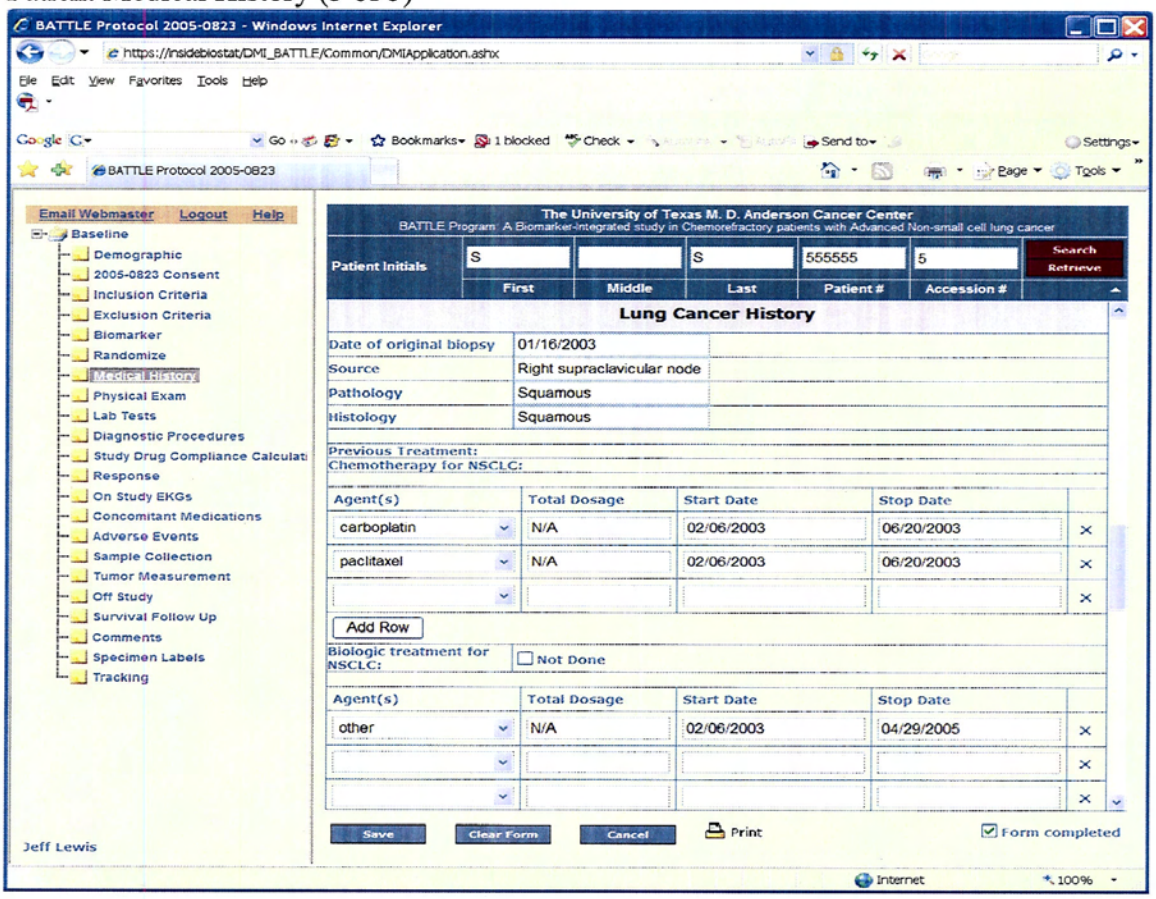
Patient Medical History (1 of 5)



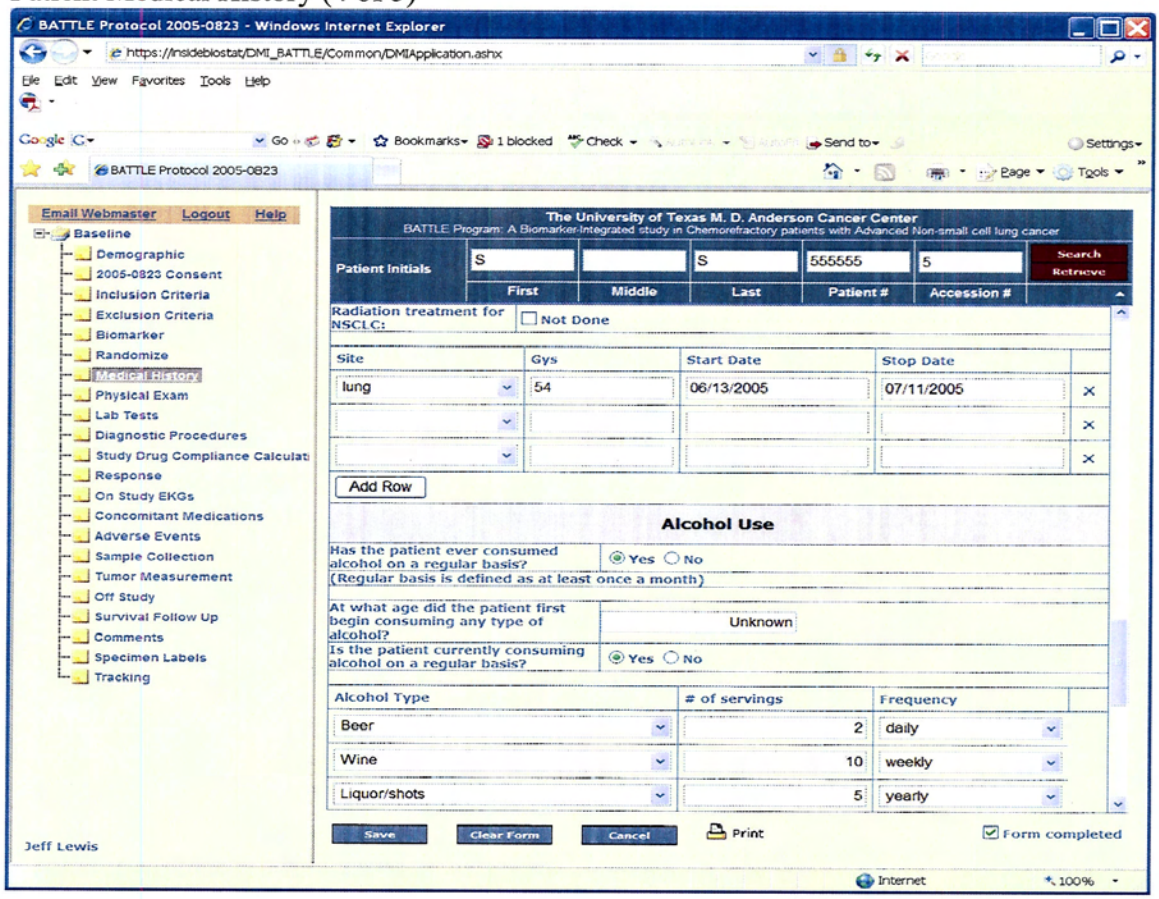
Patient Medical History (2 of 5)



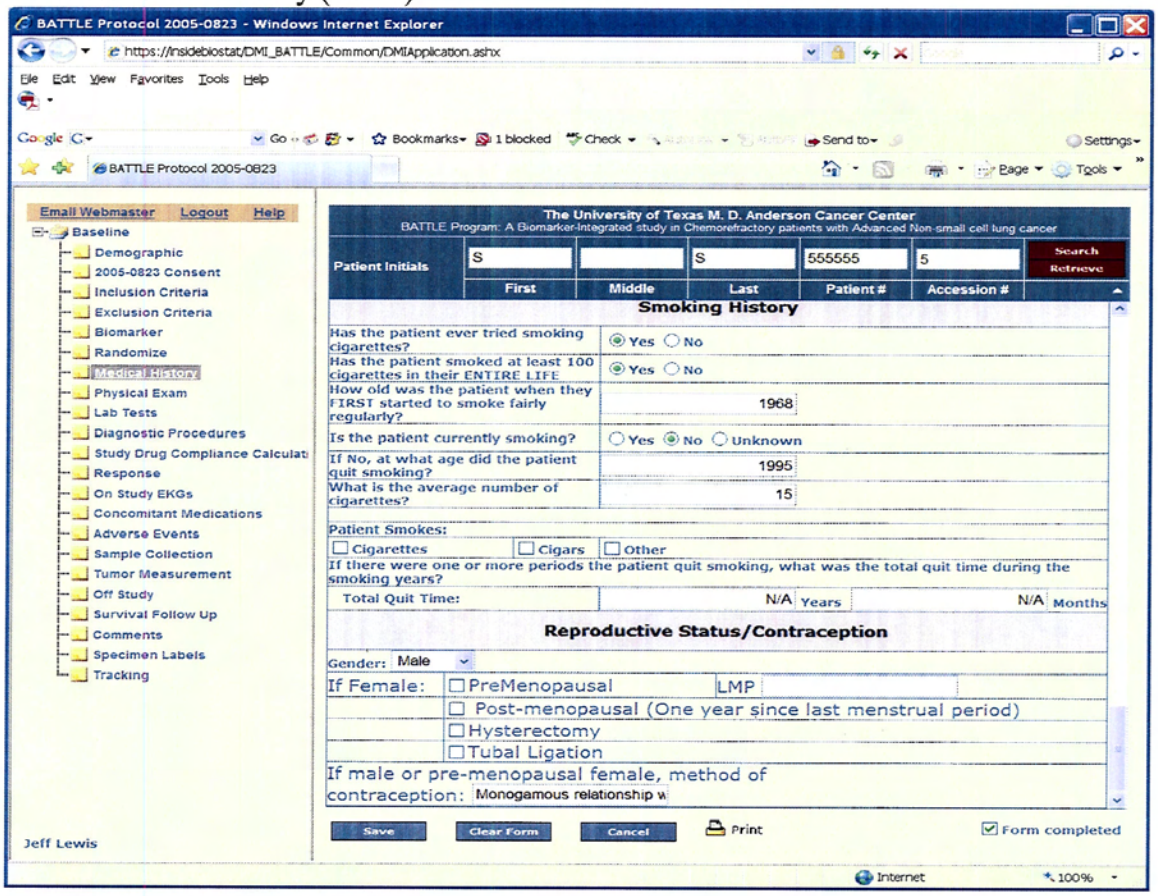
Patient Medical History (3 of 5)



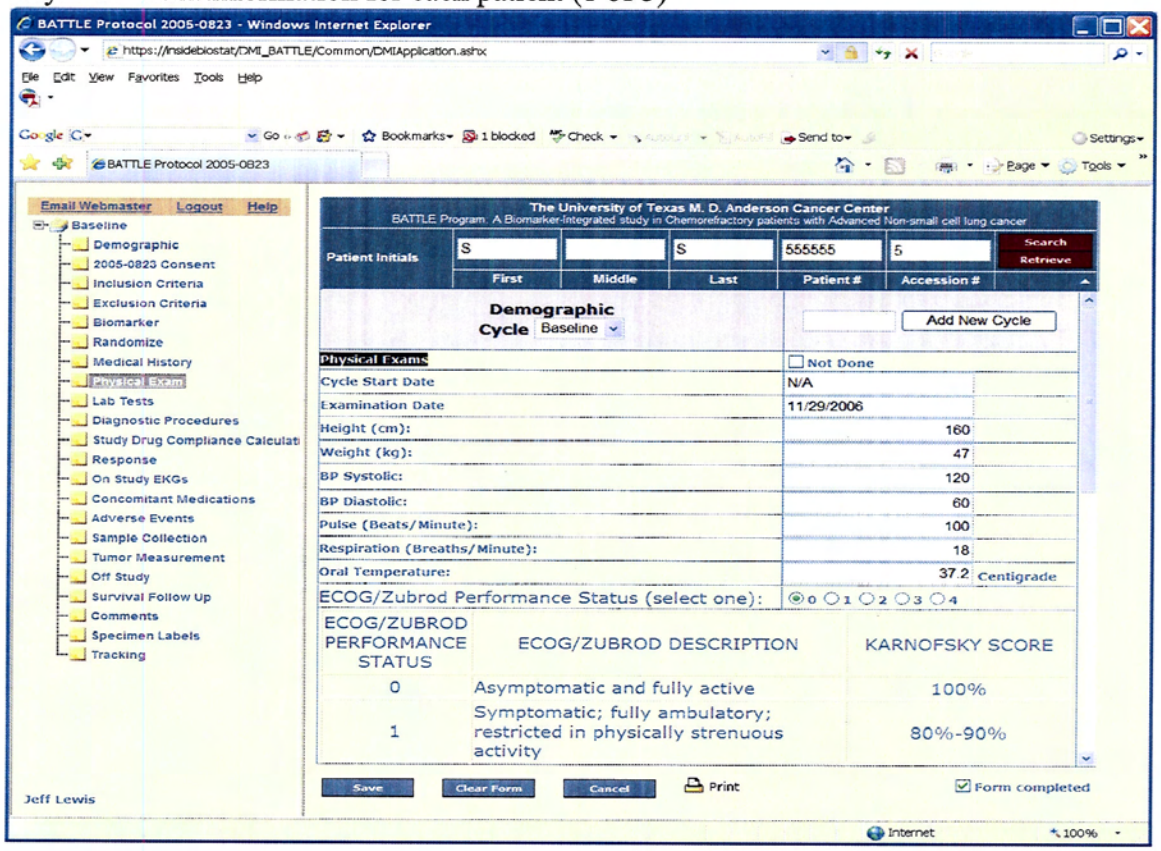
Patient Medical History (4 of 5)



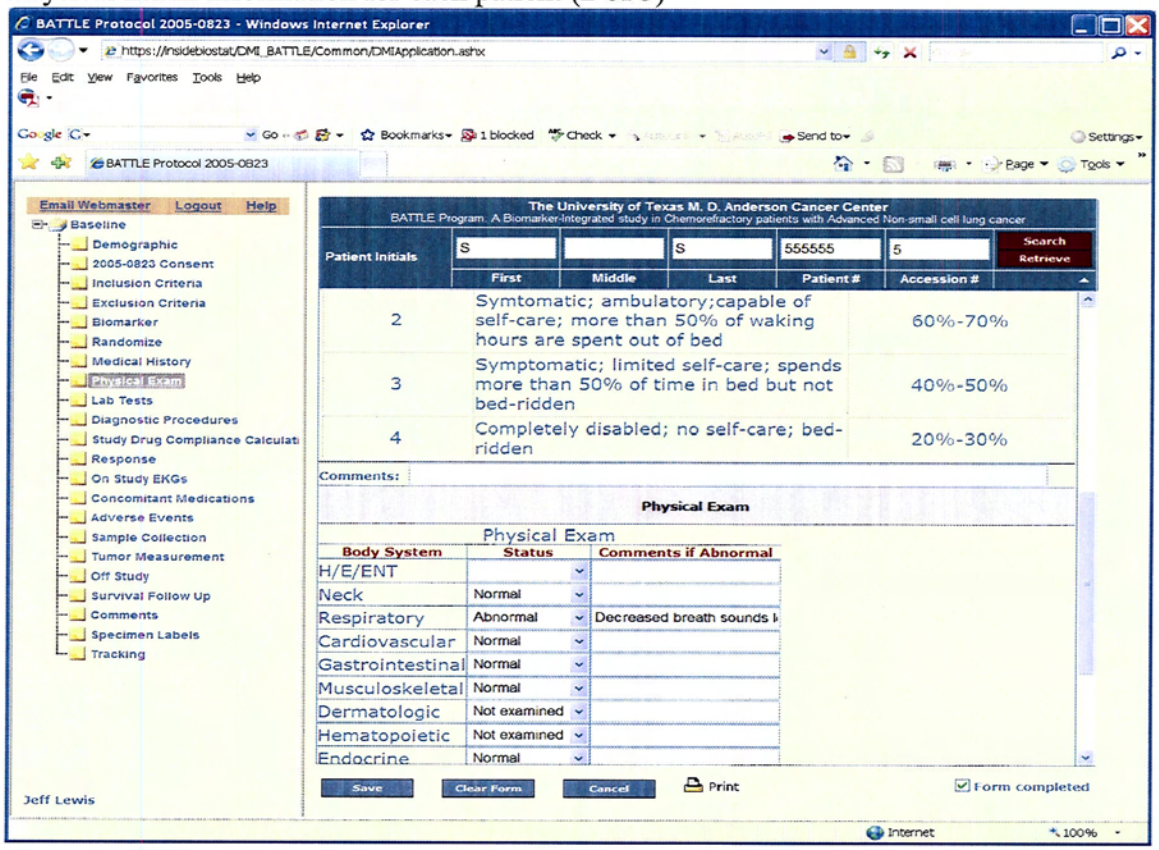
Patient Medical History (5 of 5)



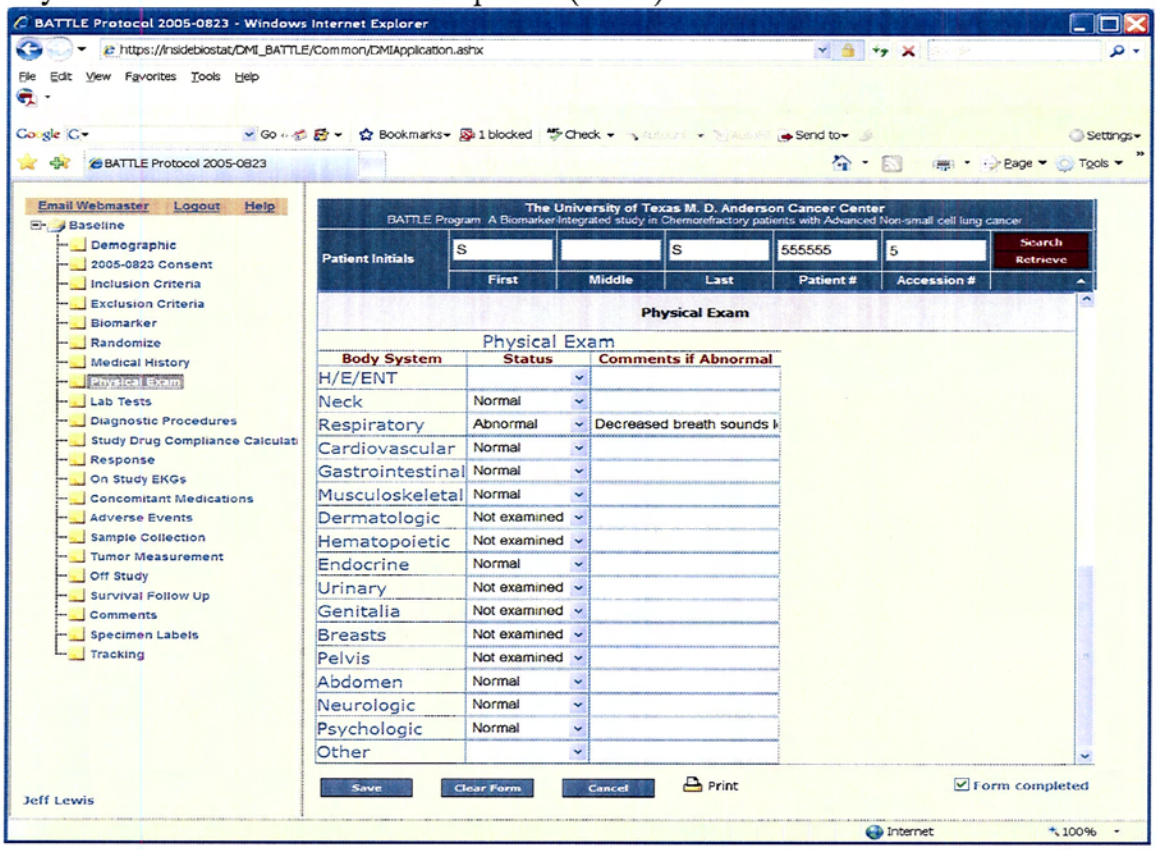
Physical Exam information for each patient (1 of 3)



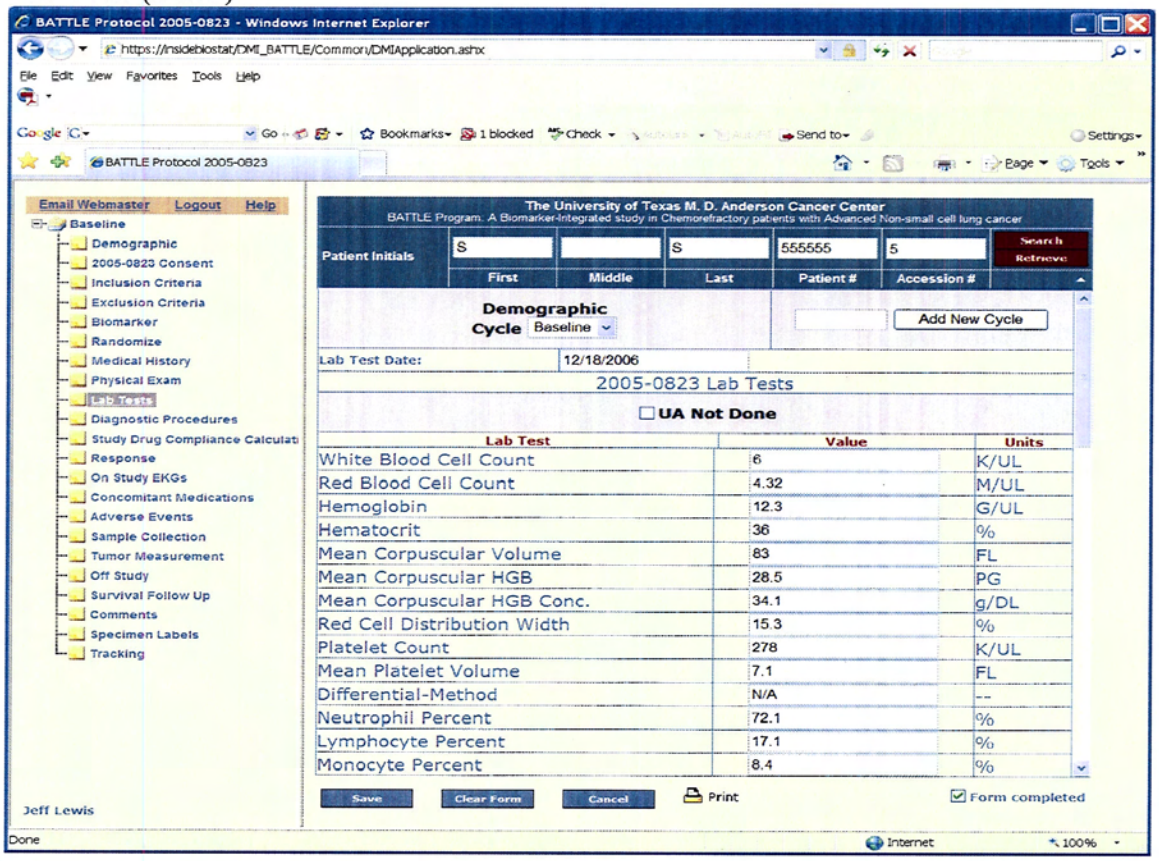
Physical Exam information for each patient (2 of 3)



Physical Exam information for each patient (3 of 3)



Lab Tests (1 of 3)



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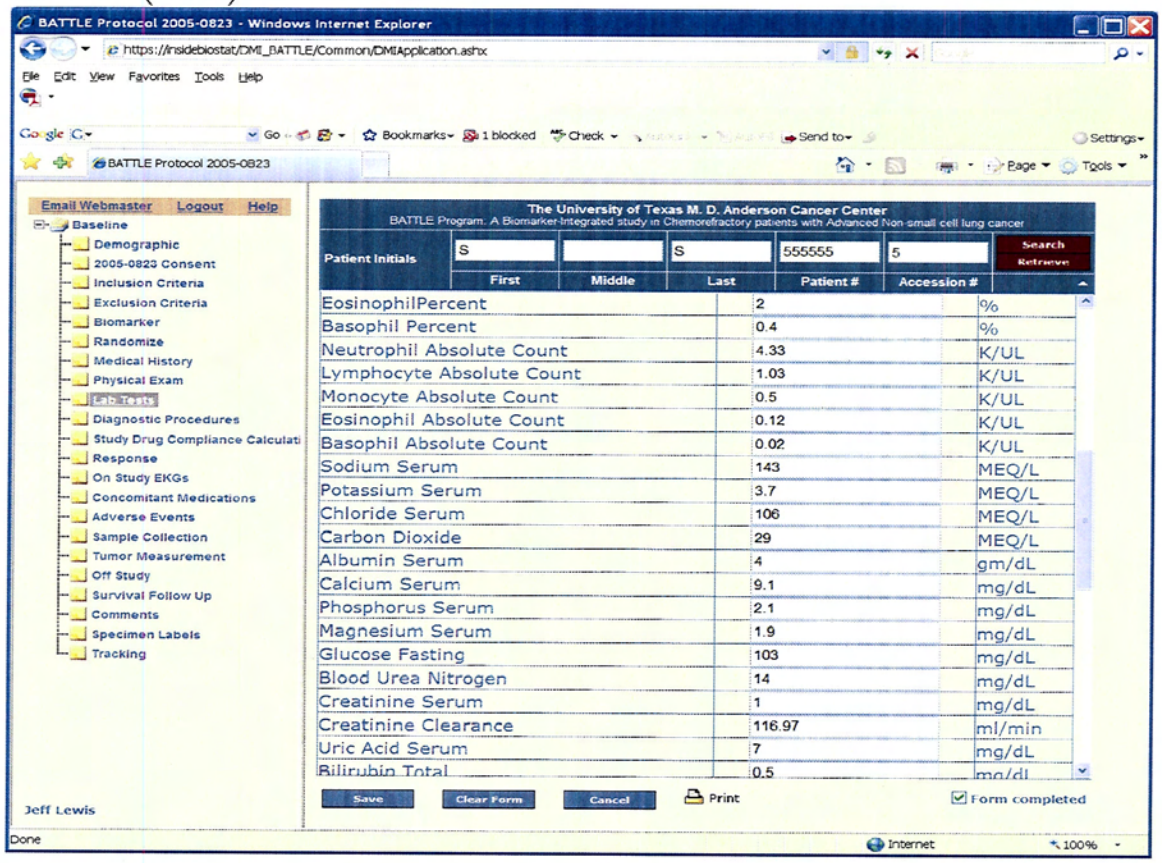
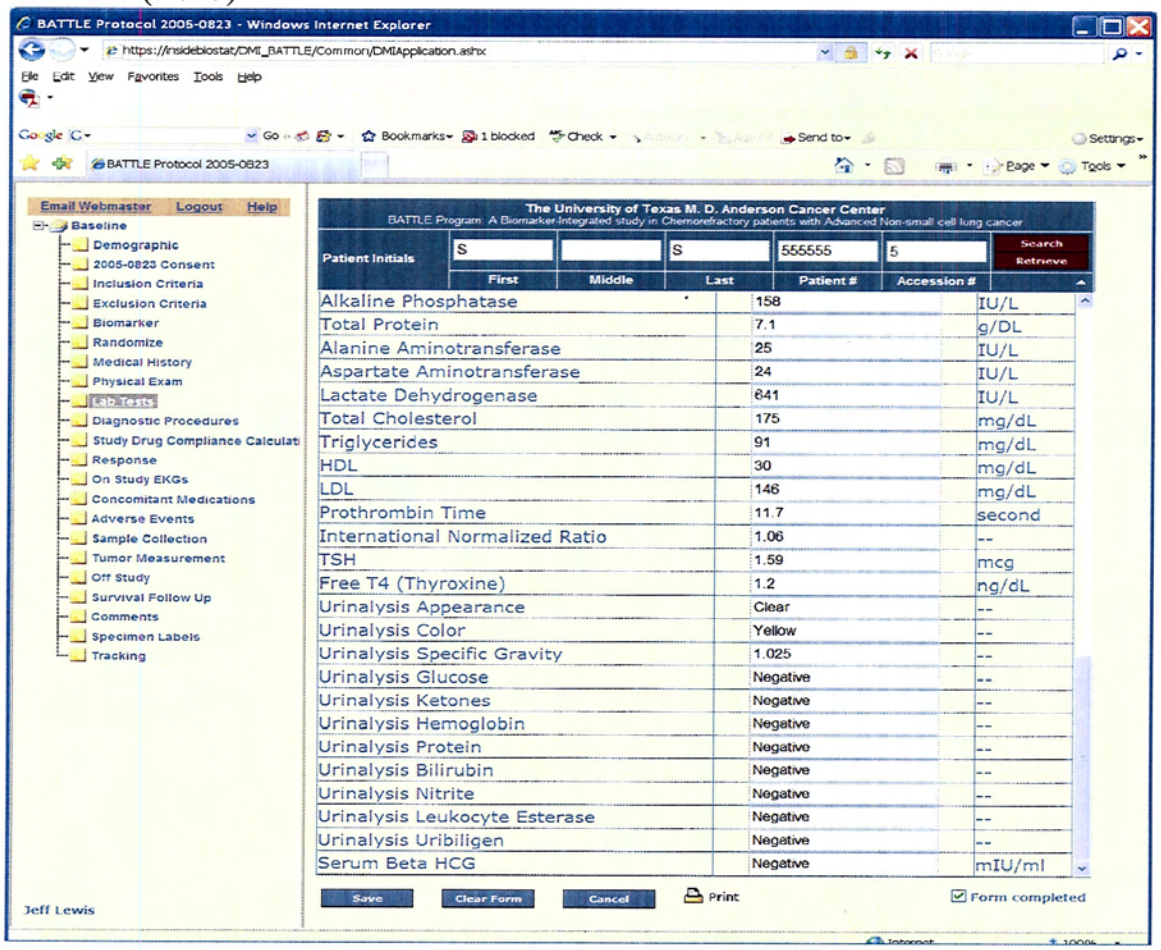
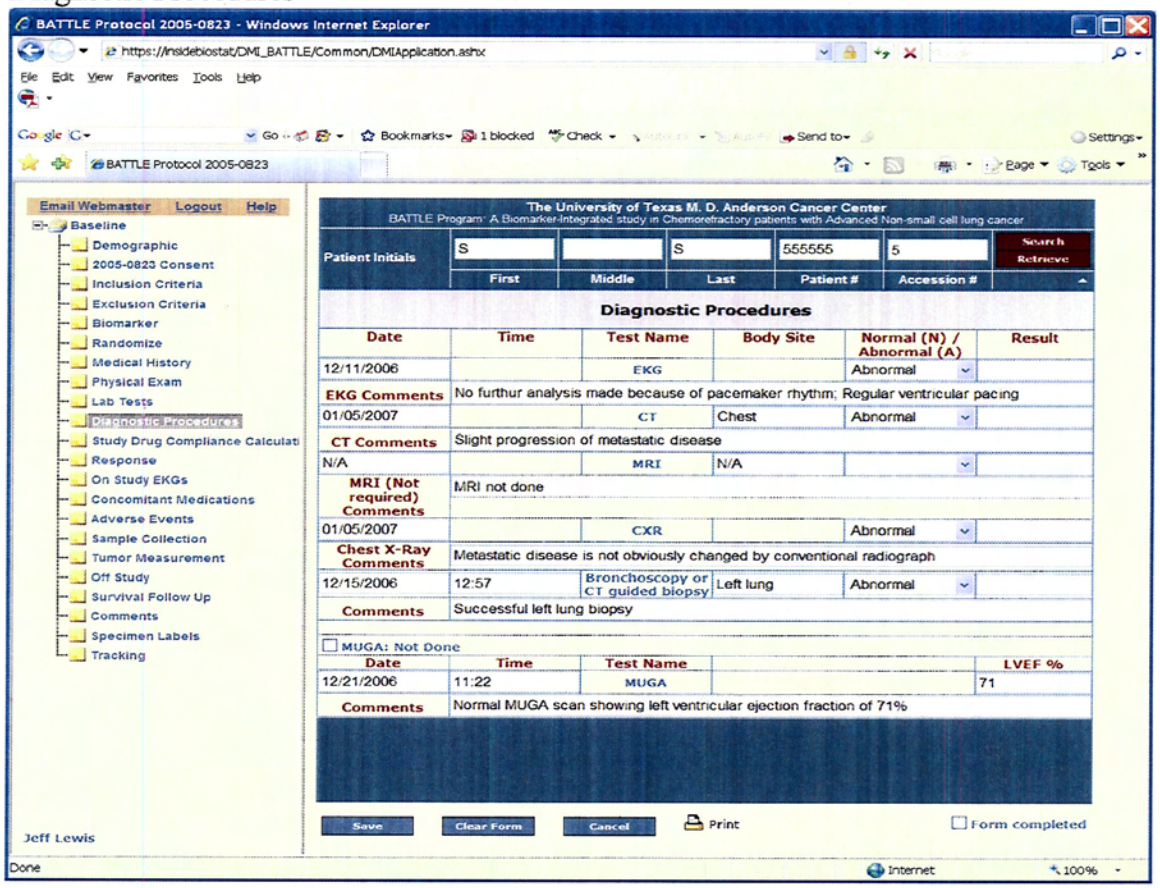


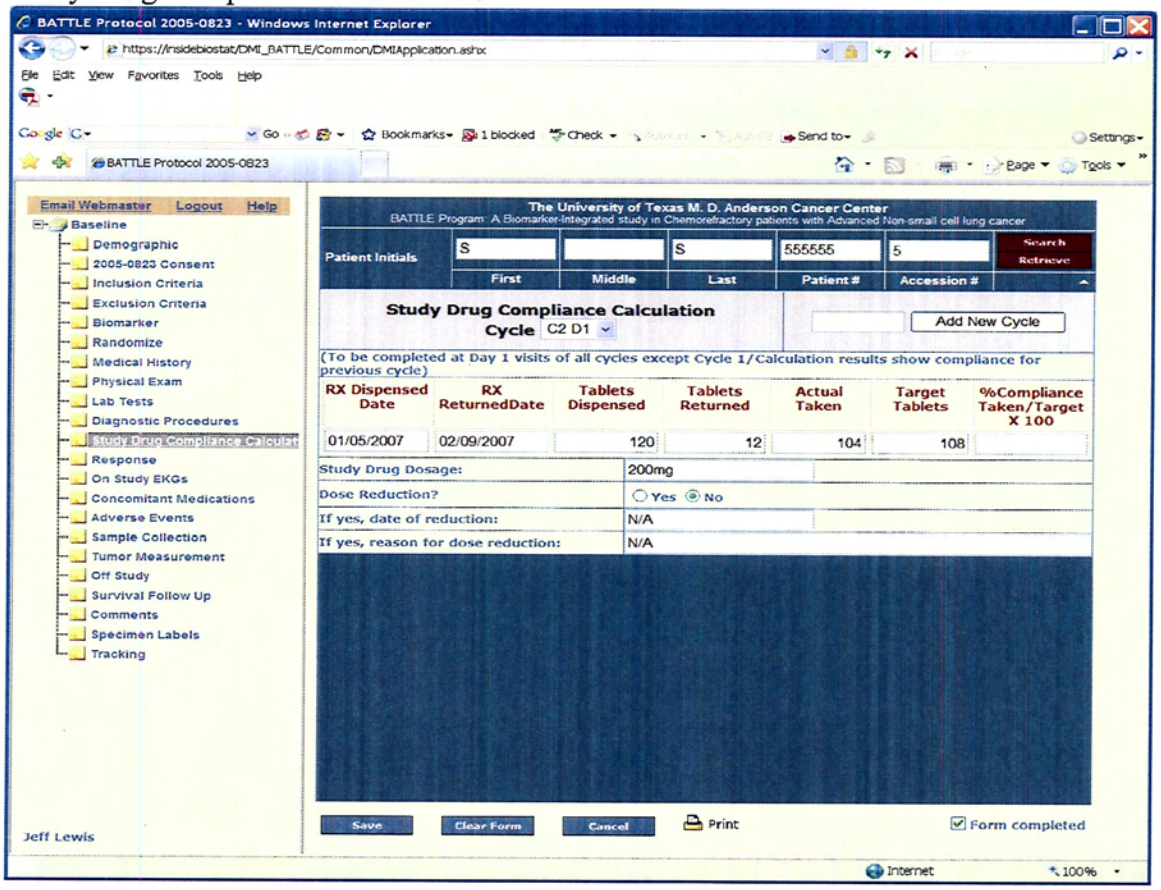
Lab Tests (3 of 3)



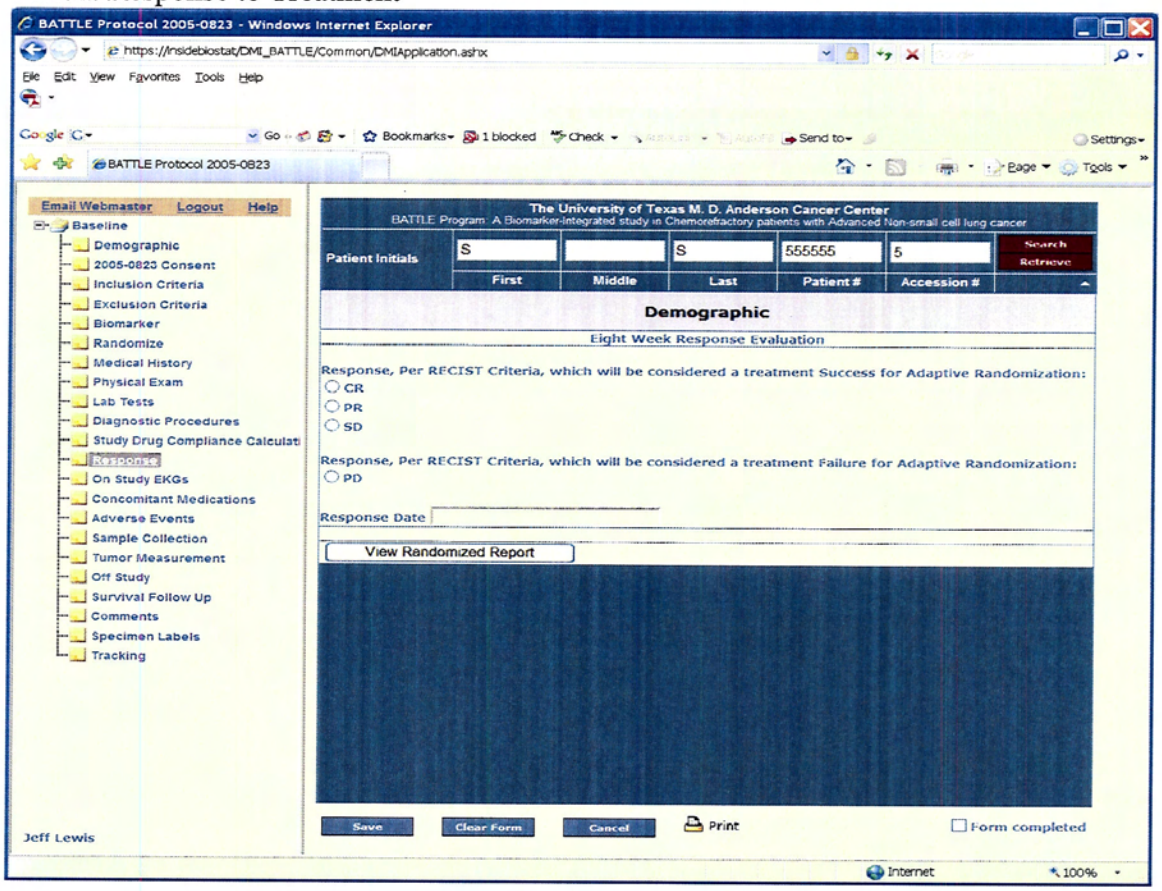
Diagnostic Procedures



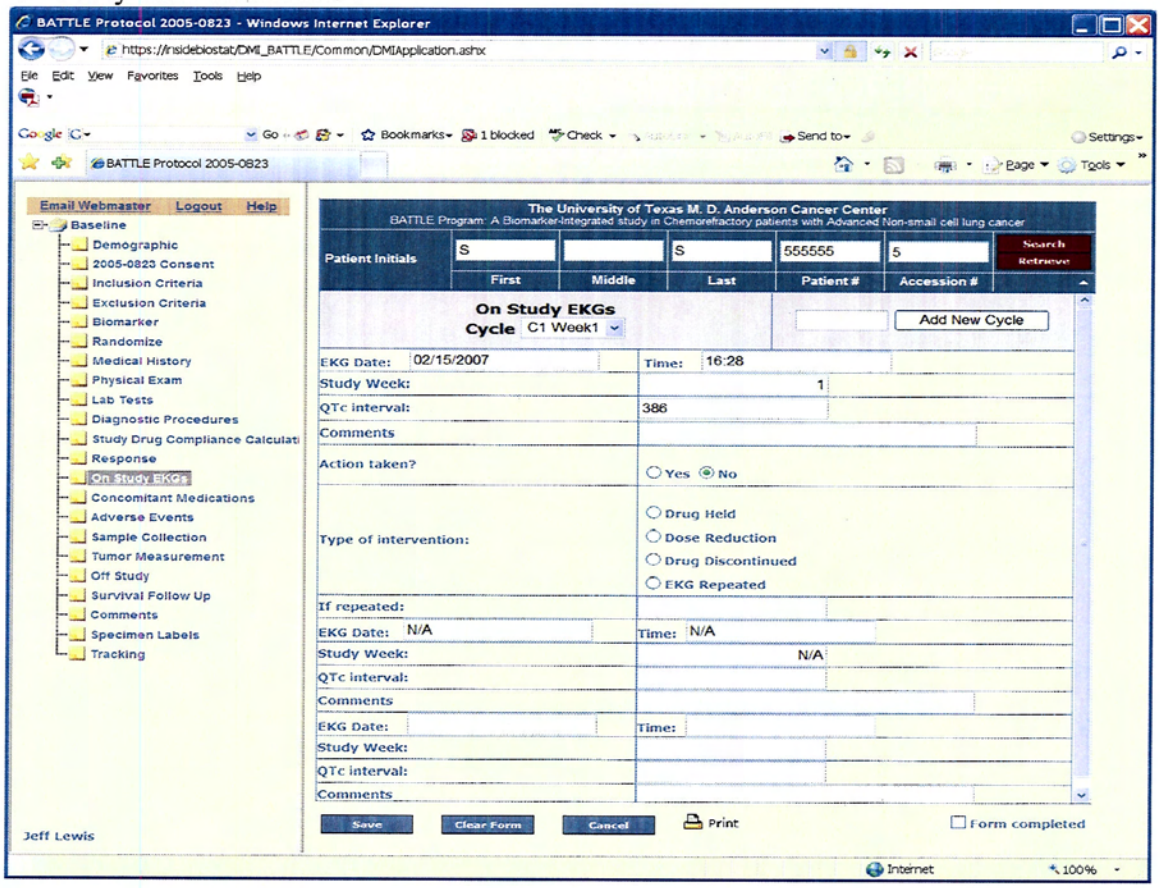
Study Drug Compliance Calculations



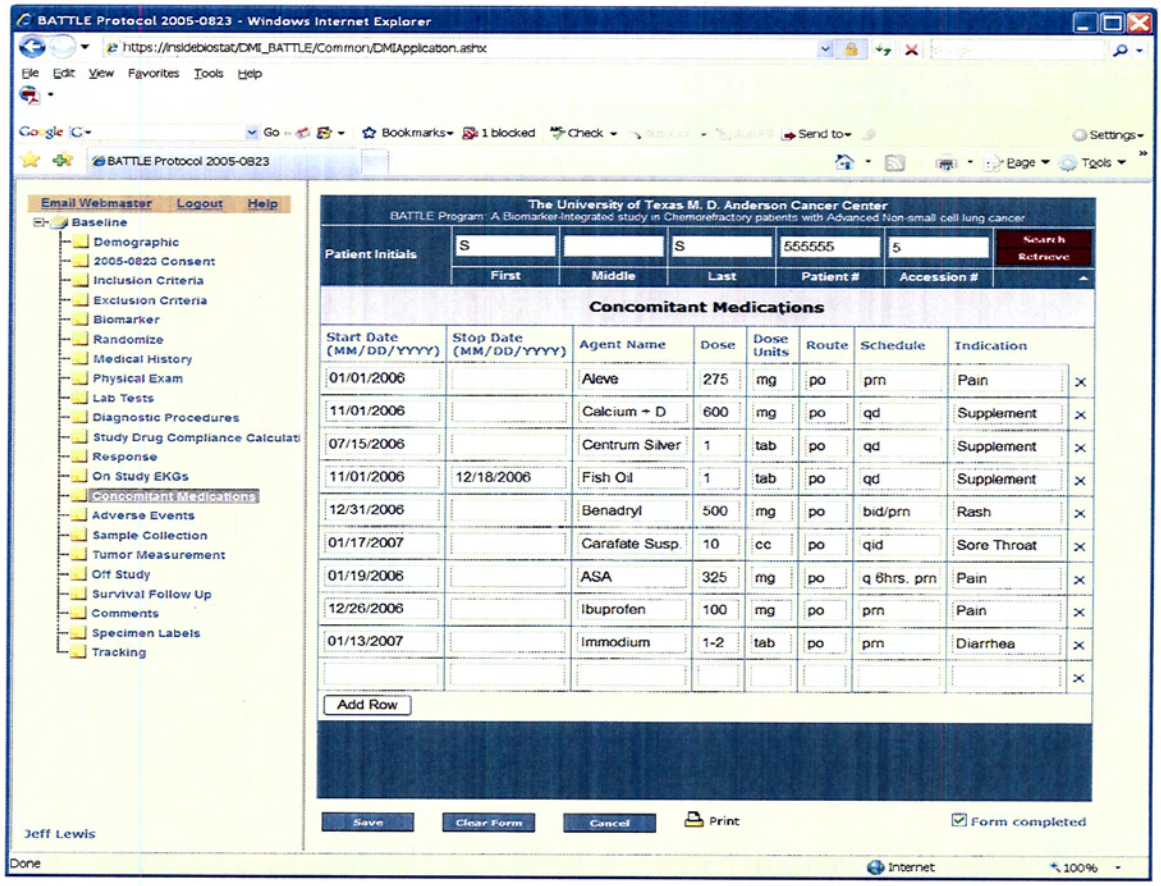
Patient Response to Treatment



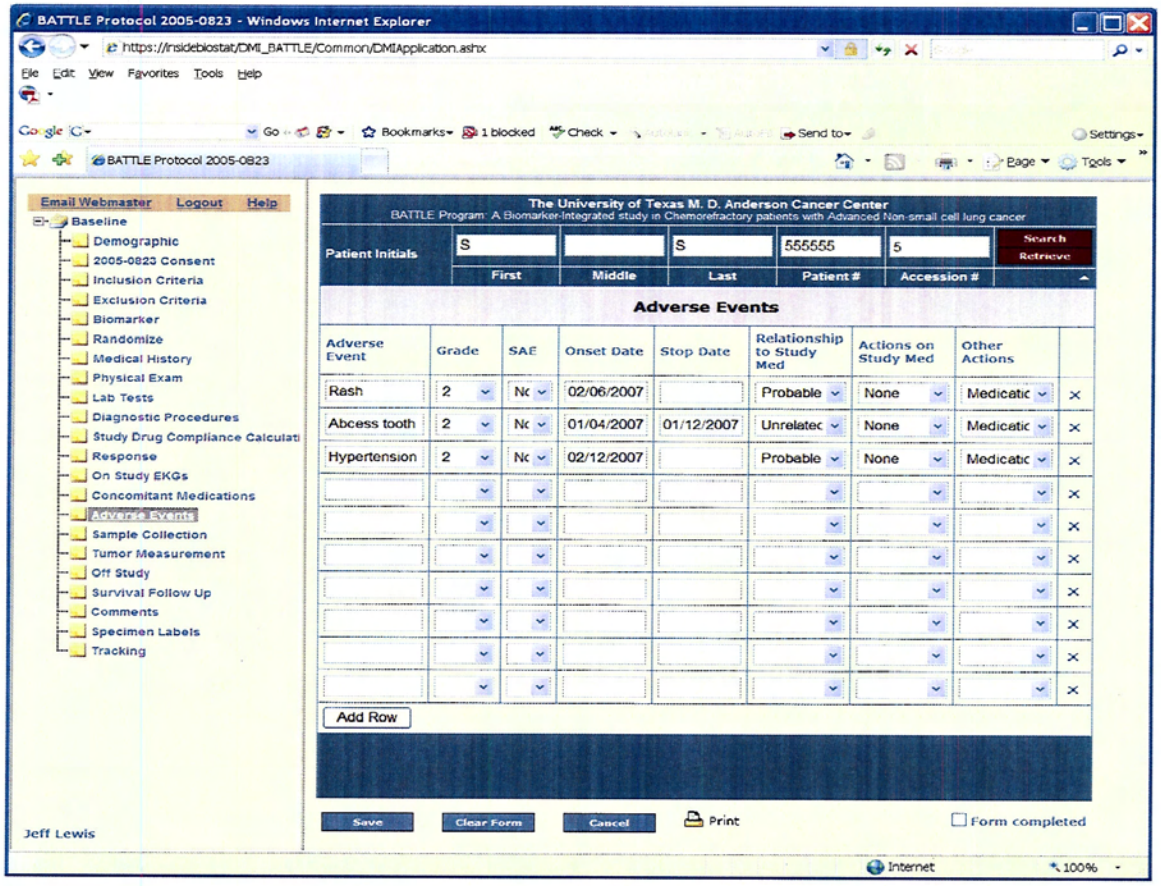
On Study EKG information



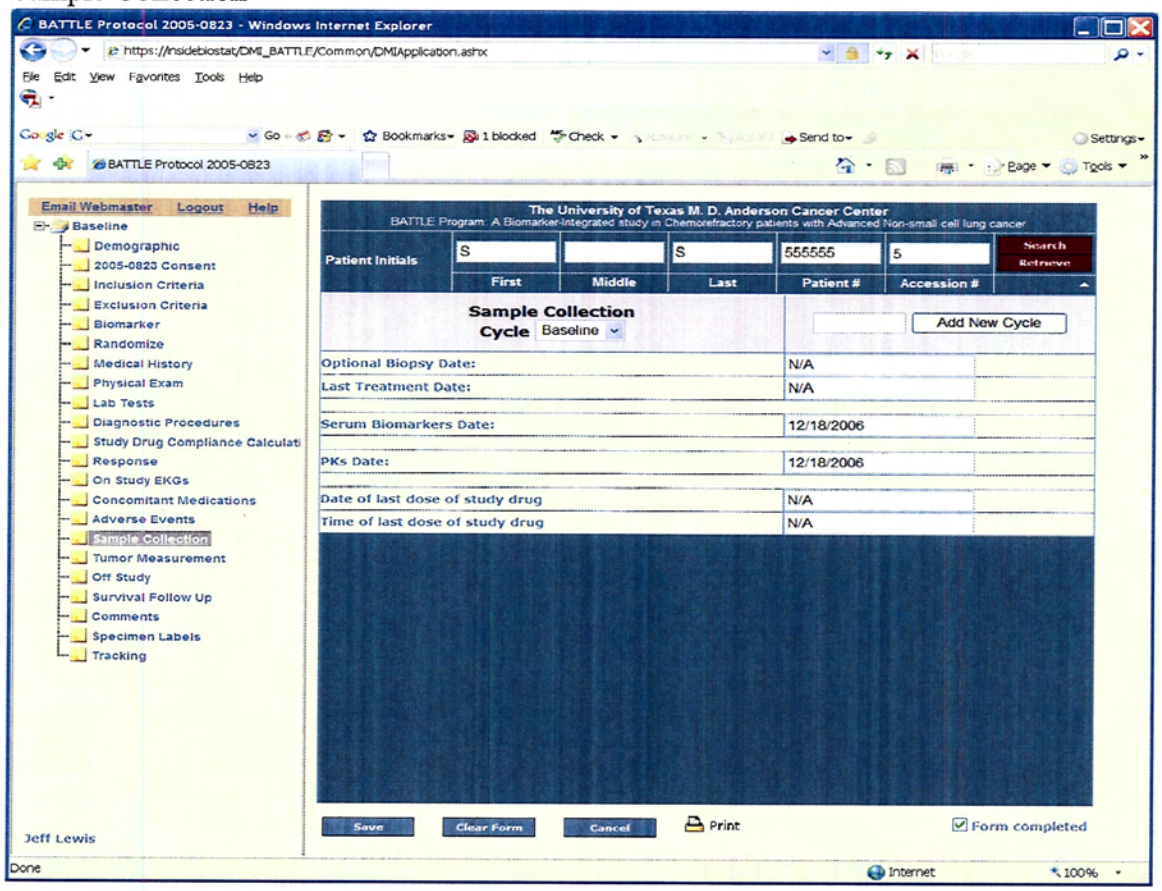
Concomitant Medications



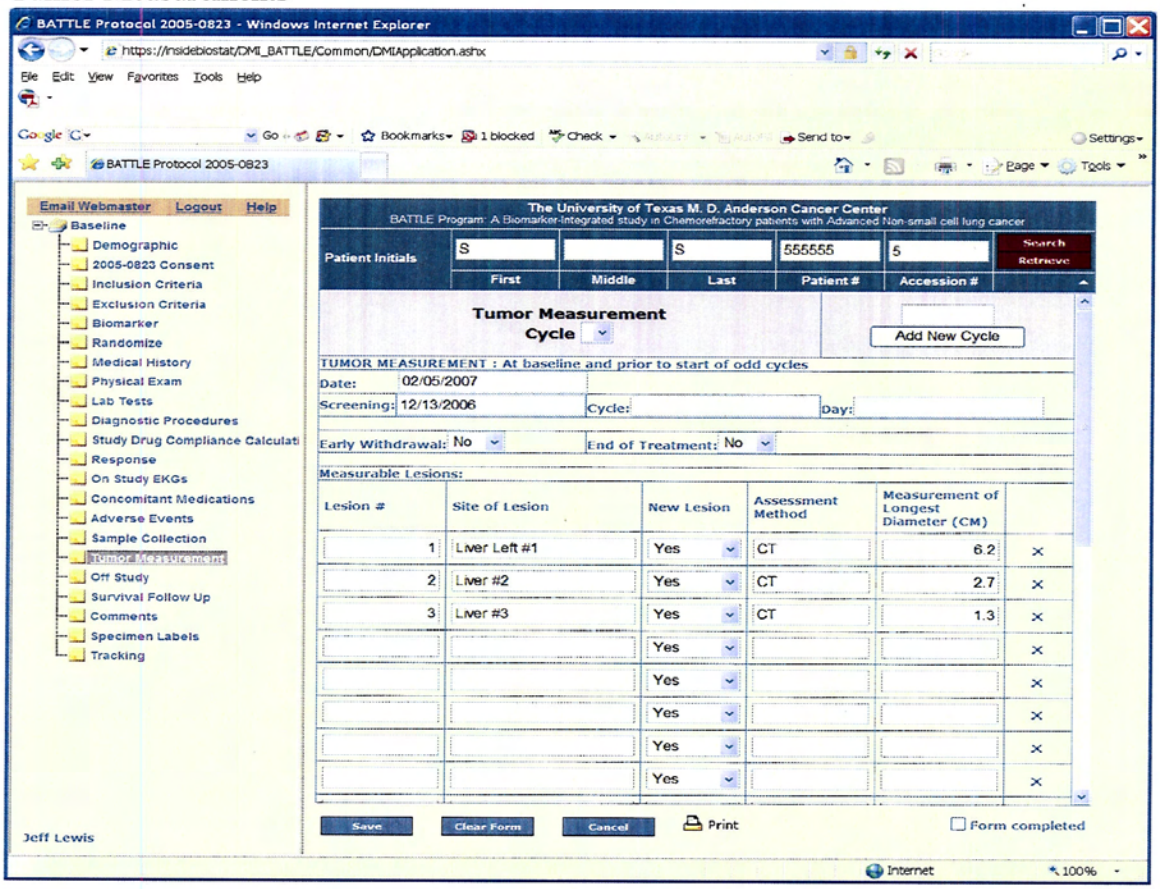
Adverse Events



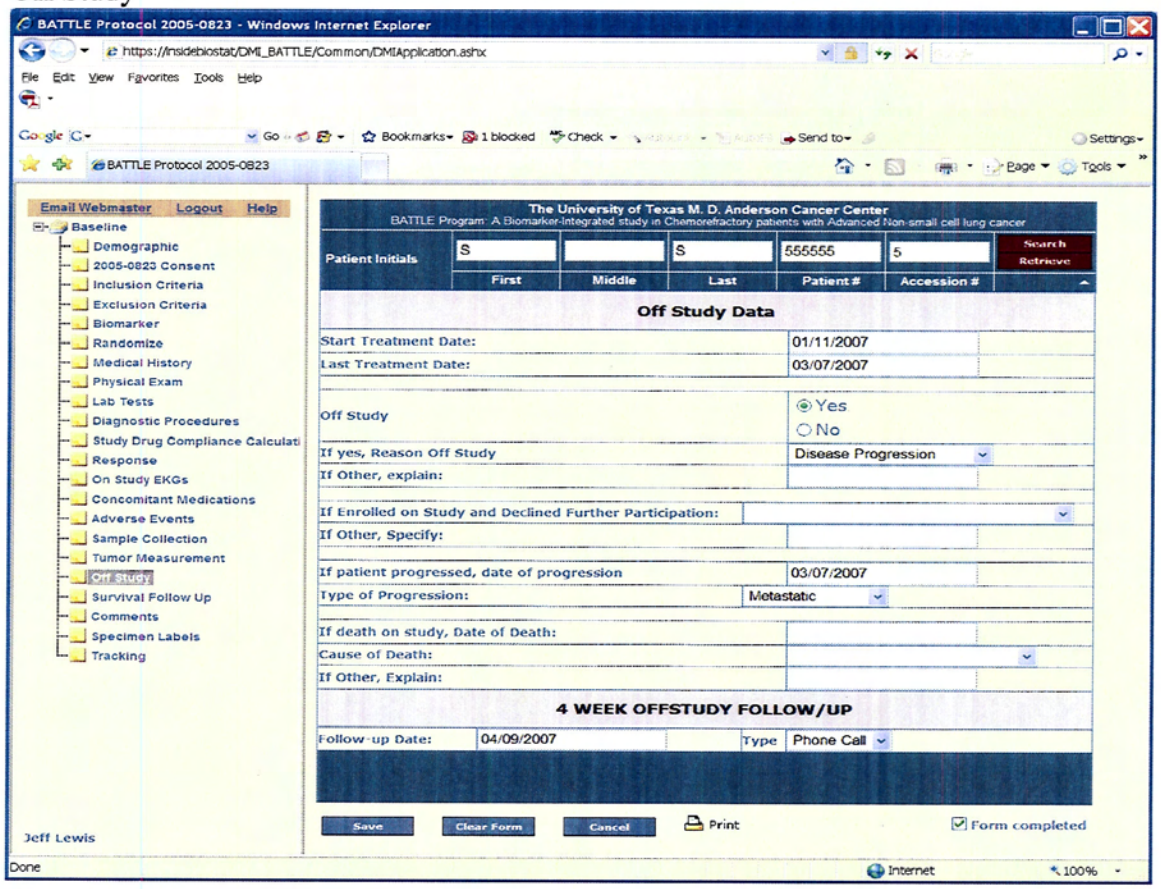
Sample Collection



Tumor Measurements



Off Study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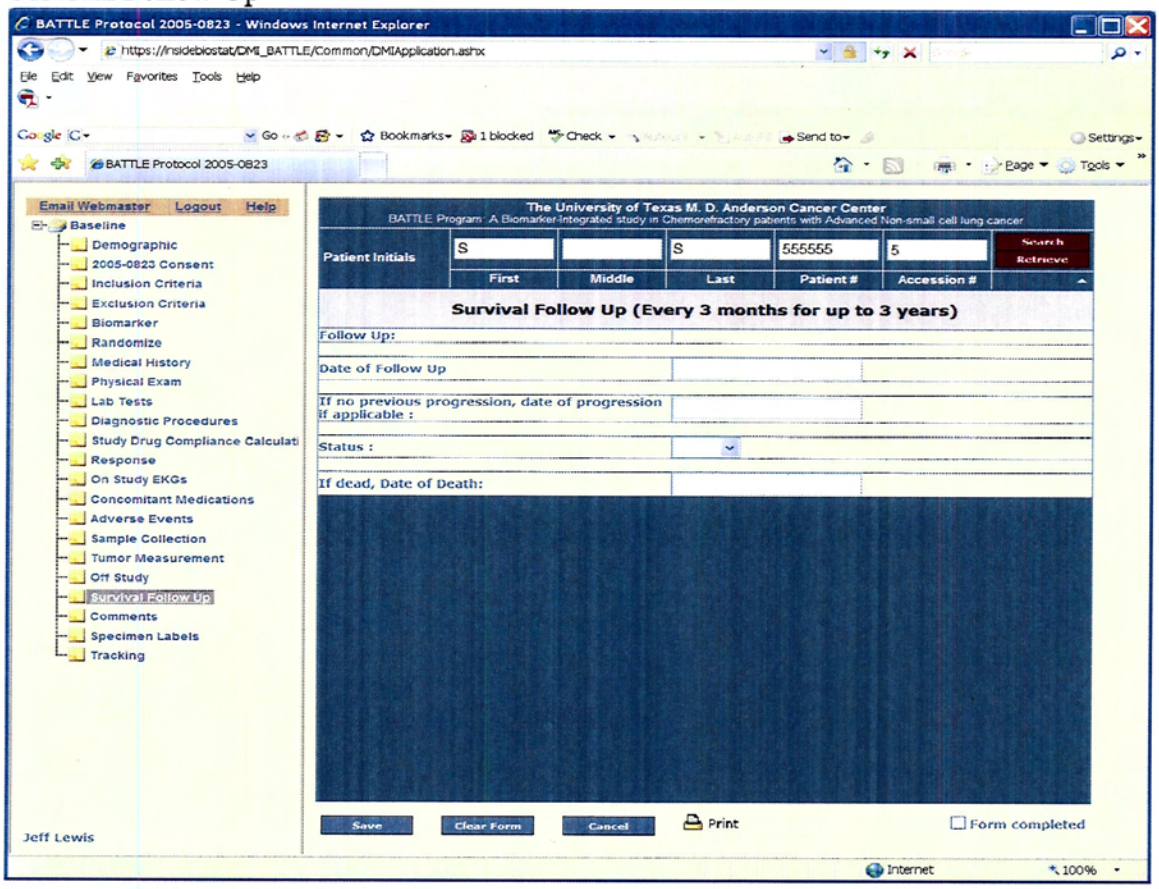
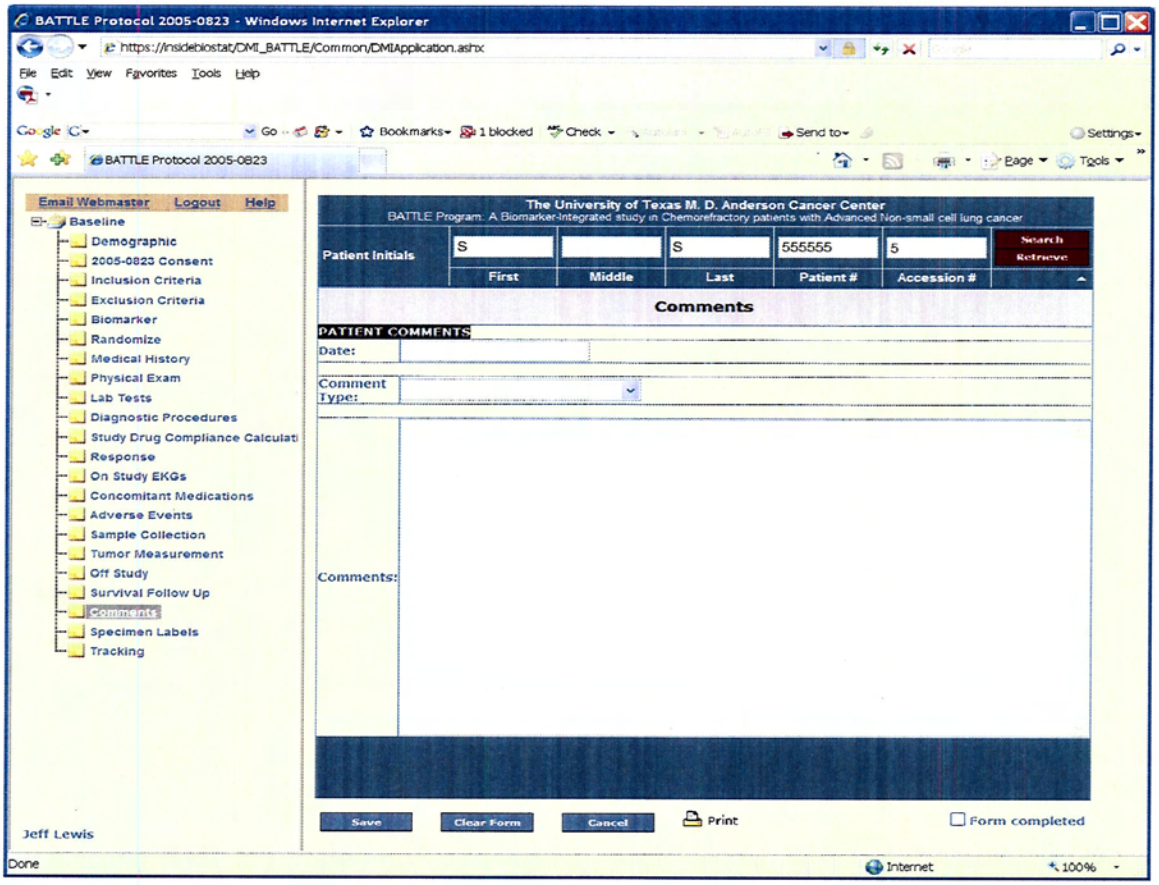
File Edit View Favorites Tools Help
BATTLE Protocol 2005-0823
Email Webmaster Logout Help
Baseline
Demographic
2005-0823 Consent
Inclusion Criteria
Exclusion Criteria
Biomarker
Randomize
Medical History
Physical Exam
Lab Tests
Diagnostic Procedures
Study Drug Compliance Calculati
Response
On Study EKGs
Concomitant Medications
Adverse Events
Sample Collection
Tumor Measurement
Off Study
Survival Follow Up
Comments
Specimen Labels
Tracking
Jeff Lewis
The University of Texas M. D. Anderson Cancer Center
BATTLE Program: A Biomarker-Integrated study in Chemorefractory patients with Advanced Non-small cell lung cancer
Patient Initials
S
S
555555
5
Search
Retrieve
First
Middle
Last
Patient #
Accession #
Off Study Data
Start Treatment Date:
01/11/2007
Last Treatment Date:
03/07/2007
Off Study
Yes
No
If yes, Reason Off Study
Disease Progression
If Other, explain:
If Enrolled on Study and Declined Further Participation:
If Other, Specify:
If patient progressed, date of progression
03/07/2007
Type of Progression:
Metastatic
If death on study, Date of Death:
Cause of Death:
If Other, Explain:
4 WEEK OFFSTUDY FOLLOW/UP
Follow-up Date:
04/09/2007
Type
Phone Call
Save
Clear Form
Cancel
Print
Form completed
Done
Internet
100%

Survival Follow Up



Comments


BATTLE Protocol 2005-0823 - Windows Internet Explorer
https://insidebiostat/DMI_BATTLE/Common/DMIApplication.ashx
File Edit View Favorites Tools Help
BATTLE Protocol 2005-0823
Email Webmaster Logout Help
Baseline
Demographic
2005-0823 Consent
Inclusion Criteria
Exclusion Criteria
Biomarker
Randomize
Medical History
Physical Exam
Lab Tests
Diagnostic Procedures
Study Drug Compliance Calculati
Response
On Study EKGs
Concomitant Medications
Adverse Events
Sample Collection
Tumor Measurement
Off Study
Survival Follow Up
Comments
Specimen Labels
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PATIENT COMMENTS
Date:
Comment Type:
Comments:
Save
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